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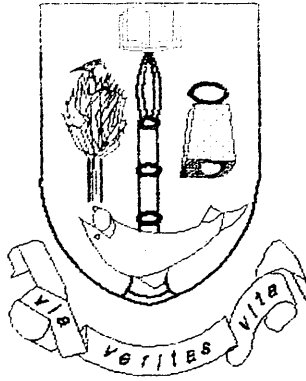
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**FUNCTIONAL RESPONSE OF
NEUTROPHILS IN THE PRESENCE
OF LPS:
ASPECTS OF LOCOMOTION AND
METABOLISM IN NORMAL AND
DIABETIC
CELLS.**

By

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Thesis submitted to the University of Glasgow for
the degree of Doctor of Philosophy in the Faculty
of Medicine.

February 1990

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LIST OF ABBREVIATIONS

The following abbreviations have been used in this thesis:

2-D	Two-dimensional
3-D	Three-dimensional
<	Less than
>	Greater than
Arg-	Arginine
Asp-	Asparagine
C	Centigrade
C3b	Cleavage product of the third component of the complement system
C3bi	Cleavage product of the third component of the complement system that can bind to CR3
C3dg	Broken down product of C3bi
C5a	Cleavage product of the fifth component of the complement system
Ca ²⁺	Calcium cation
Cl-	Chlorine anion
CR1	Complement receptor type 1
CR3	Complement receptor type 3
DMSO	Dimethyl sulphoxide, grade I
ELAM-1	Endothelial-leukocyte adhesion molecule-1
Fc	Crystallizable fragment of immunoglobulin
FMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
g	Acceleration of gravity
Gly-	Glycine
gm	Gram
GM-CSF	Granulocyte-macrophage colony stimulating factor
gp63	Glycoprotein 63
H ₂ O ₂	Hydrogen peroxide
HbA1	Glycosylated haemoglobin
HBSS	Hanks balanced salt solution
HLA DQ	Human leukocyte antigen DQ
HLA DR3	Human leukocyte antigen DR3
HLA DR4	Human leukocyte antigen DR4
HOCl	Hypochlorous acid
Hr	Hour
HSA	Human serum albumin
ICAM-1	Intercellular adhesion molecule-1
IDDM	Insulin-dependent diabetes mellitus
IgG	Immunoglobulin G
IL-1	Interleukin-1
K _{av}	Constant available in column chromatography
kD	Kilo dalton
LAD	Leukocyte adhesion deficiency
lit	Litre
log	Logarithm
LPS	Lipopolysaccharides
LTB ₄	Leukotriene B ₄

M	Molar (moles per litre)
MAb	Monoclonal antibody
mg	Milligram
Mg ²⁺	Magnesium cation
min	Minute
ml	Millilitre
mm	Millimetre
mmol	Millimoles
MOPS	3-(N-morpholino) propanesulphonic acid
MPO	Myeloperoxidase
M _r	Molecular weight
mV	Millivolt
NaOH	Sodium hydroxide
ng	Nanogram
NIDDM	Non-insulin-dependent diabetes mellitus
NK	Natural killer
nm	Nanometre
O ₂	Oxygen
O ₂ ⁻	Superoxide anion
OH ⁻	Hydroxyl anion
OZ	Opsonized zymosan
PAF	Platelet activating factor
pH	Negative logarithm of hydrogen ion
PMA	Phorbol myristate acetate
PMN(s)	Polymorphonuclear(s)
PMNL	Polymorphonuclear leukocyte(s)
prf	Phenol red-free
Pro-	Proline
R-	Rough
RGD	Triplet sequence of amino acid
S-	Smooth or wild type
Ser-	Serine
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
ug(μg)	Microgram
ul(μl)	Millilitre
um(μm)	Micrometre
v/v	Volume per volume
V _e	Elution volume
V _o	Void volume
V _t	Total volume
α	Alpha
β	Beta
≥	Greater than or equal to

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Summary

This thesis reports a study of effects of bacterial lipopolysaccharides (LPS) on morphological polarization, locomotion and chemiluminescence of human neutrophil leukocytes (polymorphonuclear leukocytes:PMN).

Change from a spherical to a polarized morphology by a cell is the initial event in cell locomotion. PMN in suspension were shown to change shape even in the presence of small amounts of LPS (≥ 100 ng/ml) in the medium. Exposing PMN to LPS from rough strains of bacteria resulted in slow shape change (60-90 minutes), unlike chemotactic factors which cause shape change within a few minutes. This suggests an indirect effect. Rough strains were more active than the smooth chemotypes in inducing polarization of PMN. The number of polarized cells increased as the period of incubation increased implying the possible presence of a second (non-LPS) agonist that promoted further polarizing activity. Polymyxin B sulfate (10 μ g/ml) was added to the cells prior to challenging with LPS (10 μ g/ml) in order to inhibit this biological effect. However, polymyxin in this system did not reduce the number of polarized PMN to a statistically significant extent. To test if a chemotactic-type factor might have been released by the cells, supernatant was prepared from PMN (10^6 cells/ml) which had been incubated with LPS for 90 minutes. The supernatant (but not LPS alone) was shown

to cause the shape change within 10 minutes in a short-term polarization assay on a new batch of PMN. By reducing the cell density in the presence of constant amounts of LPS (10 $\mu\text{g/ml}$), it could be shown that polarizing activity of the supernatant was reduced.

To assess the relationship of shape change to that of locomotory ability, three additional leukocyte locomotion assays were employed. With a micropore filter assay, migration of the cells in different concentration gradients of the factor showed that the released material behaved like a chemotactic factor as analyzed by the checkerboard system. A collagen gel assay was used to study detailed cell locomotion quantitatively (population of cells migrating) and also qualitatively (morphological changes during locomotion). Detailed behavioural analysis of the PMN response to LPS was obtained from visual studies. Quantitative measurements of shape change showed a slowly developing increase in elongation of the cells exposed to LPS for up to 90 minutes, in contrast to rapid elongation of cells exposed to the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (FMLP).

One of the ways a neutrophil might release a stimulant is through arachidonic acid metabolism. Steroidal and non-steroidal antiinflammatory drugs used to modulate the shape change process of the leukocytes displayed varied effects. Two drugs, with reported

inhibitory effects on lipoxygenase activity, BW755C and Revlon 5901A reduced the number of PMN polarized by LPS. Conversely, neither indomethacin nor dexamethasone had any effect. Preliminary characterization of the cell released factor was also conducted. The factor was heat stable and non-dialyzable. Hexane extraction, thin layer chromatography and column gel filtration suggested that the released material was heterogenous. A low molecular weight factor (about 2000 kD) was identified by gel filtration on Sephadex G-25.

Monoclonal antibodies against the CD11/CD18 family of cell surface receptors (shown by other researchers to be responsible for the binding of LPS to PMN) were used as inhibitors of LPS binding to PMN. Some antibodies were relatively effective in inhibiting LPS-induced PMN polarization particularly those against CD11b/CD18. Combinations of monoclonals against three antigens (CD11a, b and c) were more effective than any one alone. The study using these antibodies unexpectedly showed that the binding site of LPS to the phagocyte is highly affected by antibodies to the α -chain and not the β -chain as reported by previous investigators. Competitive inhibitory binding of LPS to PMN was observed with synthetic peptides containing an RGD-sequence. The observation points to a probable involvement of the sequence in LPS-induced neutrophil polarization.

Chemiluminescence was used as an additional test of LPS activation of PMN. On addition of LPS (10 $\mu\text{g/ml}$), slow but progressive light emission was noted. Normal peaking time ranged from 30 to 60 minutes with different intensities from different donors. Addition of platelet activating factor (PAF) as a primer did not enhance the response but instead slightly depressed the activity. LPS on its own can also act as a priming agent. Preexposing cells to LPS for 30 minutes at 37° C followed by adding PMA gave enhanced chemiluminescence. Similar priming but using opsonized zymosan as a second stimulus however depressed the activity. Comparison of the priming effect with PAF shows that PAF potentiates both responses to soluble and particulate stimulus. The degree of chemiluminescence differs between the two primers with the different stimulus used.

Results obtained from normal individuals were used as standards to compare with diabetic patients both in polarization and chemiluminescence studies. Diabetic PMN showed poor responses in a polarization assay. With the addition of PAF as a potentiator, the response increased. Chemiluminescence using LPS alone, opsonized zymosan and PMA was comparatively lower in diabetics than in normals. The presence of LPS or PAF as primers

for the responses towards opsonized zymosan and PMA increased the activity. There was no positive correlation between the response to LPS and levels of blood glucose, fructosamine or glycosylated haemoglobin in individual patients.

CHAPTER 1

INTRODUCTION

The inflammatory process can be triggered by bacterial invasion or by physiological changes in the body such as physical trauma or immunological reactions. The inflammatory process is manifested by three distinct features which generally lead to an end result of restoration of normal function. Nevertheless, there are instances whereby severe injury can be initiated as a consequence of the inflammation. The three main features of inflammation can be summarised as follows:-

- A) the enlargement or dilatation of the elastic blood vessels hence allowing a large volume of blood flow to the injured area.
- B) the increased permeability of the vessel walls to plasma proteins which are normally retained in the blood vessel.
- C) the migration of polymorphonuclear leukocytes (neutrophils) and monocytes into the extravascular space. The process usually involves certain systematic characteristics. Leukocytes initially adhere to the endothelial cell lining. Having adhered, the cells traverse the vessel wall actively, using an amoeboid pattern, or motile towards the inflammatory stimulus.

The latter feature in inflammation has been the subject of intensive research for the past twenty years. Substantial evidence has thus far emerged in understanding the emigration and accumulation of

leukocytes in vivo. Endotoxins, lipopolysaccharides derived from the Gram-negative bacteria are among the important mediators which cause these leukocytes to accumulate at the site of injury (Issekutz and Bhimji, 1982; Issekutz and Megyeri, 1987). Bacterial lipopolysaccharides (LPS) elicit local inflammation when injected into an animal, but the role of lipopolysaccharide in inducing the infiltration of leukocytes into the inflammatory lesion has received much more attention than its other inflammatory effects. Leukocyte membrane proteins which mediate adhesion to endothelium have recently been discovered to contribute to the extravasation of these inflammatory cells to the extravascular space. Lipopolysaccharide is known to enhance this adhesion as discussed later. Information to support this notion is presented by the discovery of patients with leukocyte adhesion deficiency (LAD) lacking adhesion glycoproteins on the cell surface of neutrophils (Springer et al, 1984; Arnout et al, 1984).

With the availability of quantitative assay to assess the mechanisms and kinetics of leukocyte migratory behaviour, a better picture of the actual situation in the inflammatory process should be displayed soon. It is therefore hoped that the work in this thesis will be of help in solving the puzzle of leukocyte locomotion and accumulation.

Section 1

Early studies of inflammatory cells in inflammation

Inflammation was clearly recognised in ancient Egypt as recorded by the historians. During the nineteenth century, extensive work was conducted to better understand the process. Of particular interest was the phenomenal involvement of leukocytes sticking to the blood vessels following injury. Dutrochet in 1824 was recorded as the first who observed leukocyte sticking to the vessel wall and their emigration thereafter (Grant, 1974), but Addison and Waller were widely accepted to be among the pioneers who documented the event clearly (Movat, 1985). Some twenty to thirty years later other investigators made considerable progress in their initial work. Among those forefront figure was Cohnheim, in 1889, whose contribution to the study of cellular emigration was extensive. He is quoted as mentioning that the veins were filled with innumerable colourless corpuscles that did not remain entirely motionless but advanced slowly. These observations were noted in the mesentery and tongue of the frog. The movement of these leukocytes through a vessel wall was however considered by Cohnheim to be a passive motion and he referred to it as a filtration process (Cohnheim, 1889). This was countered by later workers particularly Elie Metchnikoff (Metchnikoff, 1893) who

strongly believed in the active participation of the leukocytes. By the turn of the century, it was generally known that leukocytes marginate, adhere to the endothelium and eventually emigrate. The precise events during migration of the leukocytes were elucidated with the advent of electron microscopy and the in vitro studies of leukocyte locomotion and chemotaxis.

Chemotaxis was first described in leukocytes by Leber in 1888 (Movat, 1985) when he injected *Aspergillus fumigatus* in the cornea of rabbits. Leber described the directional movement of leukocytes outside vessels as due to the differences in concentration of the substance with which these cells come into contact. The term "chemotaxis" was used by Bloch in his 1896 publication to indicate such activity as observed by Leber (Movat, 1985). Interpretations of the earlier findings on chemotaxis were later well understood when chemoattraction of PMN was shown to be indispensable in the Arthus reaction (Ward and Cochrane, 1965) due to complement activation by immune complexes. However, chemotaxis as the principal mechanism in in vivo leukocyte accumulation was not fully recognised until after the in vitro experiments of Boyden (Boyden, 1962). Thus the study of leukocyte

chemotaxis received a considerable impetus with the description of a chamber by Boyden. A new field of "chemotactic movements" was consequently explored to further strengthen the understanding of leukocyte accumulation at the site of inflammation.

Section 2

Polymorphonuclear leukocytes

In the early days of microscopic anatomy, it was discovered that blood and tissues of animals contained large numbers of cells that were capable of locomotion and showed a tendency to engulf foreign materials from their environment. These cells were thought to function as scavengers but also to be responsible for spreading infections by engulfing and transporting microbes in the tissues. In the late 1890s, Metchnikoff discovered that these cells, phagocytes as he called them, were not just scavengers but would engulf and kill the most common bacteria. Hence, he had shown that the phagocytes constituted one of the principal agencies of defense against microbial invaders. Phagocytes may be divided into two general classes depending on their nuclear structures:-

- (i) polymorphonuclear cells with a segmented nucleus of two or more lobes
- (ii) mononuclear cells possessing a single kidney-shaped nucleus.

The former class forms the subject of the whole dissertation. The designation, polymorphonuclear leukocytes or granulocytes, is not specific to the cells under discussion since it includes basophils, neutrophils, and eosinophils. The accepted term in

dealing with the specific cell type discussed in this project is the neutrophil leukocyte. It is the most common granulocyte accounting for approximately two-thirds of the white cells in human blood.

Morphology

The cell is well identified in Wright-stained blood smears as having a multilobulated, dark-staining nucleus and a pinkish cytoplasm filled with small granules. The cell diameter is 10 micrometer and the lobes are connected to one another by very thin strands of nuclear material. One of the most unusual features of these cells is the striking nuclear shape. The molecular mechanism for the development and maintenance of the divided nucleus is still completely unknown. Metchnikoff suggested that the unique structure of the nucleus would facilitate the passage of these cells through the capillary walls. A notable feature of neutrophil ultrastructure is the rarity of organised cytoplasmic structures other than the granules. The cells seem to lack a nucleolus or ribosomal aggregates and have little or no smooth or rough endoplasmic reticulum, a small Golgi apparatus and few small sized mitochondria (Thompson, 1977; Laszlo and Rundles, 1986).

Granules

As has been mentioned, one of the _____ distinguishing features of neutrophils is the abundance

of cytoplasmic granules that are usually centered in the cell. Two types of granules have been identified in man: azurophil or primary granules and specific or secondary granules (Bainton et al, 1971). The former comprise one-third of the population displaying primarily intracellular lysosomal contents while the latter are more accessible to extracellular release. Primary granules appear first about the promyelocyte stage. They store the majority of the neutrophil's potent antimicrobial components, hence fulfilling the requirements of the cell's role in host defense. Myeloperoxidase is the protein that is responsible for the peroxidase activity, characteristic of azurophilic granules. In the presence of hydrogen peroxide and halide, together they function as the cidal mechanism of the target cells (Klebanoff, 1980). Lysozyme is a cationic enzyme that has been shown to kill certain Gram-negative and Gram-positive microorganisms via enzymatically hydrolysing the bacterial cell wall and also by a non-enzymatic mechanism; although for several years it has been proposed that the primary bactericidal spectrum of lysozyme is limited to saprophytic gram-positive organisms such as *Micrococcus* and *Bacillus* species (Salton, 1957; Selsted and Martinez, 1978). Bactericidal/permeability-increasing protein, a cationic bactericidal protein, exerts selective bactericidal action against gram-negative bacteria non-

enzymatically (Elsbach and Weiss, 1985). The polypeptide isoenzyme cathepsin G, one of the neutral serine proteinases, exhibits chymotrypsin-like esterase activity and has a broad antimicrobial spectrum of action on gram-positive and gram-negative bacteria which is independent of enzymatic function (Odeberg and Olsson, 1975; Odeberg and Olsson, 1976; Shafer et al, 1986). The major constituent of primary granules, representing 5% to 7% of total cellular protein in neutrophils, is the antimicrobial peptides, defensins (Ganz et al, 1985; Selsted et al, 1985). These structurally and functionally homologous peptides are small (M_r 3500-4000), cysteine-rich, moderately cationic and identical except at their amino terminal residues. Recently, three carbohydrate-free defensins have been identified (Rice et al, 1987). The proposed mechanism of antimicrobial actions is by permeabilizing the inner and outer membranes of the bacteria (Lehrer et al, 1989). The spectrum is broad including gram-positive and gram-negative bacteria, certain viruses and fungi (Daher et al, 1986; Lehrer et al, 1988). Most of the primary granule products are released late in inflammation from dead and dying neutrophils and may have important role in inhibiting the extent of inflammation. Thus, in addition to amplifying inflammation, primary granule secreted products may be vital in turning off the inflammatory response (Gallin, 1984).

Later in maturation, the synthesis of primary granules is checked (Bainton, 1973). The secondary granules rich in glycoprotein then develop and predominate in the matured cells. Even though specific granules contain lysozyme as do azurophilic granules, they also contain characteristic components such as lactoferrin and vitamin B₁₂-binding proteins. Specific granules are readily mobilised during neutrophil activation appearing to fuse with phagosomes before the azurophil granules do (Bainton et al, 1971). During the migration of neutrophils to sites of inflammation, fusion of specific granules with the plasma membrane, accompanied by releasing of contents to the extracellular environment, takes place. Lactoferrin is an iron-binding protein which may block bacterial growth by reducing a free-iron concentration to below the required level needed by all bacteria to proliferate (Oram and Reiter, 1968). The protein has also being observed to facilitate production of hydroxyl radicals (Ambruso and Johnston, 1981) and may regulate myelopoiesis (Broxmeyer et al, 1980). The role of vitamin B₁₂-binding protein is not known.

Origin and development of neutrophils

Neutrophils are produced mainly in the bone marrow at a rate of 2.5 billion cells per hour from the precursor stem cells. These stem cells are capable

of differentiating to several cell lineages and in vitro were termed spleen colony forming units (CFU-S) (Quesenberry et al, 1979). Differentiation of the CFU-S leads to the generation of the precursors of matured neutrophils. Regulation of proliferative activity of these stem cells is ascribed to several growth factors including interleukin 3 whilst the production of neutrophils is driven by growth factors known as granulocyte macrophage colony stimulating factor or GM-CSF (Sieff, 1987). Maturation of neutrophils has been described by several authors (Ackerman, 1964; Bainton et al, 1971; Jandl, 1987).

Myeloblasts: This is the earliest morphologically recognisable cell of the series. It occurs in normal bone marrow comprising up to 5% of the white cells and is only found in circulation in disease states. Myeloblasts are round mononuclear cells with a large purplish-blue nucleus in Wrights stain smears. The cytoplasm may contain azurophilic structures that have staining and ultrastructural characteristics of azurophil granules. The myeloblast then differentiates into a promyelocyte.

Promyelocytes: Older cells at this stage are larger than the myeloblast with numerous azurophilic granules in the cytoplasm and in the region overlaying the

nucleus. However, these granules are quite distinct from the specific neutrophilic granules.

Myelocytes: The cells succeed the promyelocytes. Specific blue-brown neutrophilic granules that stain with peroxidase appear during this stage. Myelocyte maturation can be subdivided into several stages. The two known stages are metamyelocyte ("Juvenile") and band (stab) cells. In the latter stage, the nucleus begins to elongate, folds and coils into heterogenous nuclear form that gives the band cell its name. Thus, this is the first of a neutrophilic line that can be regularly identified in normal circulating blood.

Polymorphonuclear (segmented) neutrophils: These cells are the end product of the granulocytic series which take between 6-14 days to develop. The nuclei are segmented into two or more lobes. The cytoplasm reveals great numbers of specific granules and this fully mature functional leukocyte is ready to undertake the task of combating organisms infiltrating the body.

Distribution and fate of mature neutrophils

The proliferative organ of bone marrow contains a large reserve pool of mature polymorphs that can be mobilised rapidly especially during the states of stress as in infection. Once mature neutrophils enter the peripheral blood, half of the population will be in

circulation and another half adhering to the endothelium of small vessels, margination (Cartwright et al, 1964). As morphologically matured neutrophils move from the marrow reserve pool to the circulating pool and equilibrate with the marginal pool, they acquire their utmost functional capacity. The half-life of these neutrophilic granulocytes in the blood stream is about 6 to 7 hours (Athens et al, 1961). When the cells leave the vascular space they migrate into tissues where they perform their phagocytic function within a lifespan of 4 to 5 days.

Generally it has been accepted that exhausted neutrophils meet their fate in situ since there is little evidence of their returning to the blood stream from the inflamed sites or that the lymphatics provide a major disposal route (Fliedner et al, 1964; Hurley, 1983). Recently a group of investigators has shown that with time, increasing numbers of neutrophils undergo characteristic morphological changes indicative of a programmed cell death or apoptosis. The process leads to recognition by macrophages of senescent but structurally and functionally intact neutrophils (Savill et al, 1989). Their observations may therefore represent a mechanism of neutrophil removal that is relatively important in the resolution of inflammation and limitation of tissue injury in acute inflammatory reactions .

Metabolism of neutrophils

Neutrophils utilise glucose as an energy source and store glycogen as a reserve supply in the cytoplasm. Glycolysis is the main energy production pathway as for example in phagocytosis (Sbarra and Karnovsky, 1955; Cline, 1975). The phagocytes do function well in an anerobic environment thus enabling them to control bacterial proliferation or clean up debris in necrotic tissues devoid of blood supply (Vel et al, 1984). During phagocytosis the rate of oxygen uptake is increased along with lactic acid production. Activation of NADH oxidase is also largely responsible for the increased utilisation of O_2 (Stossel et al, 1971). Production of hydrogen peroxide ensues in the course of neutrophil's metabolic activity. The tricarboxylic acid cycle and oxidative phosphorylation play a minor role in energy production, hence the relative paucity of mitochondria in mature neutrophils. The cells show essentially little or no RNA synthesis, have no amino acid pool in the cytoplasm and barely any DNA synthesis. They do, however, exhibit active metabolism and rapid turnover of lipids especially during phagocytosis (Williams et al, 1986).

Physiology of neutrophils

In inflammatory reactions neutrophil functions involve a sequence of steps as listed:-

A) adherence of leukocytes to capillary endothelium

A continuous physiologic interaction exists between PMN and the vessel wall in which about one-tenth of the total blood PMN pool emigrate from the bloodstream every hour by adhering and diapedesing across the endothelium (Athens et al, 1961). Following local injury, cells adhere to the side of vessel wall nearest to the site of injury but not to the undamaged endothelium in the vessels downstream when they are occasionally dislodged (Allison et al, 1955). These observations suggest that endothelium adjacent to the site of injury becomes more adhesive. There is a possible functional alteration in endothelium that promotes adhesion even though specific morphological alteration is usually not apparent (Florey and Grant, 1961). On treatment with LPS or interleukin-1 or tumour necrosis factor, vascular endothelium becomes more sticky for leukocytes and expresses increased amounts of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) or endothelial-leukocyte adhesion molecule 1 (ELAM-1) discovered by several investigators (Pober et al, 1986; Pohlman et al, 1986; Bevilacqua et al, 1987a). Regardless of whether PMN are directly stimulated by inflammatory mediators or indirectly activated by an endothelial-dependent mechanism, there is a growing interest in a functional CD18 glycoprotein which mediates neutrophil adherence to endothelium as documented by in vitro studies (Anderson

et al, 1986). Arfors et al have to a certain extent shown that membrane glycoprotein complex CD18 is indeed critical for PMN adherence and extravasation in vivo (Arfors et al, 1987). In their experiments, monoclonal antibodies recognising CD18 were injected intravenously into rabbits challenging the cellular response towards chemotactic factors that were injected intradermally. Cell accumulation and PMN-dependent plasma leakage were abolished in the inflammatory skin lesion. Intravital microscopy of rabbit muscle revealed inhibition of adherence in venules and migration into tissue following the application of leukotriene B₄, FMLP and zymosan activated serum. Rolling of PMN along the venular endothelium was found to be unaffected. Basal expression of the CD18 antigen on the neutrophil surface is important for neutrophil attachment to the endothelial cells in vivo (Price et al, 1987; Nourshargh et al, 1989).

B) emigration into the tissues

Once adherent, PMN will undergo shape changes and start to migrate actively from the vessels through the gaps between endothelial cells (Marchesi and Florey, 1960). Movement begins with an extension of a hyaline lamellipod in the direction of locomotion so that the cell becomes elongated with its long axis parallel to trajectory of motion. These characteristic morphological

alterations by the emigrating leukocytes have been documented in vitro where the cells were in suspension without any effect of a contact with planar substratum (Keller et al, 1983; Shields and Haston, 1985). It is known that neutrophils in suspension can become polarized and that they are capable of performing the same crawling-type movements as cells diapedesing from the blood vessels into the injured tissues (Smith et al, 1979; Keller and Cottier, 1981). The direction of migration can be determined by a gradient of chemotactic factors (Baum et al, 1971). This mode of directional migration accounts for the accumulation of cells at the source of gradient. Besides the presence of chemotactic factors, other mechanisms may be operative in the accumulation of PMN at inflammatory sites. The polarization response of PMN is discussed in more detail later. Close correlations between leukocyte behaviour in suspension and their subsequent ability to locomote once adhering to a substratum allows accurate prediction of the potential locomotor responses of the leukocyte.

C) phagocytosis

Having arrived at the invasion site phagocytes must recognise what to attack, and demonstrate remarkable selectivity when encountering particulate objects such as bacteria, fragments of dead cells or other foreign materials by discerning certain chemical structures

termed opsonins on the surface of objects that they ingest (Stossel, 1974). The consequence of recognition of particles coated with IgG and/or C3b via their Fc and C3b receptors is the initiation of engulfment by the phagocyte. When the object comes into contact with the plasma membrane the hyaline ectoplasm extends to form pseudopodia. The distal ends of the pseudopods finally seem to fuse so that the object is internalized by invagination and a phagocytic vacuole or phagosome is formed. Interaction between opsonized particle and plasma membrane also causes activation of oxidative metabolism of the cell whereby a variety of oxygen radicals which have strong bactericidal activity are formed (Babior, 1978).

D) degranulation

Concomitantly, the membrane of cytoplasmic granules come into contact and fuses with that of the phagosome (phagolysosome) so that the contents of granules, mostly proteolytic enzymes with bactericidal activity, are released both into the phagosomes and the surroundings of the cell (Stossel, 1974). The process is known as degranulation since granules are no longer visible as discrete structures in the cytoplasm (Hirsch and Cohn, 1960). As a result of metabolic activity and addition of contents of specific granules, the pH inside the vacuole has been reported to decrease to between 3.0 and 6.5 (Jensen and Bainton, 1973). Although many different

probes have been used, there is no clear consensus concerning pH changes in phagolysosomes. Presently, it is thought that the pH rises to almost 8.0 within few minutes after phagolysosome formation and then falls to about 6.5 within 30 minutes (Segal et al, 1981; Spitznagel, 1984). Subsequent to this, primary granules fuse with phagosome and release their content. Killing and digestion take place within the phagolysosome.

E) bactericidal action

Coincident with these dynamic events, a series of biochemical changes occur that result in the killing of ingested microorganisms. Microbicidal activity can be divided into two major categories:

(i) oxygen dependent mechanisms that rely on toxic capabilities of molecules produced via the respiratory burst.

(ii) oxygen independent mechanisms responsible for killing in an anerobic milieu or in PMN where the oxidative mechanism is deficient or absent.

Lysozyme, defensins, and cationic proteins are the main weapons in this type of killing activity.

The respiratory bursts refer to an abrupt increase in oxygen consumption that occurs when PMN are stimulated either by ingesting particles or by membrane perturbations

(Babior, 1978). Within seconds after stimulation, oxygen uptake increases and large quantities of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are released into the surrounding medium. At the same time large amounts of glucose are oxidised by the hexosemonophosphate shunt pathways. Alterations in O_2 metabolism result from the enzymatic activation of respiratory burst oxidase. Through the foregoing sequence of reactions, respiratory burst oxidase activates microbicidal agents apart from either O_2^- or H_2O_2 which are products of further reactions involving these two compounds. Two microbicidal reactants are formed by oxidation of halogens and the radicals: $H_2O_2 + Cl^- \longrightarrow HOCl + OH^-$ in the presence of myeloperoxidase (Andrews and Krinsky, 1981). Hypochlorous acid ($HOCl$) formed in this reaction is a potent microbicidal agent but unlikely to act directly in the phagolysosome. Instead reaction of $HOCl$ with ammonia and amines modulates the toxicity of this oxidant producing chloramines. Chloramines are powerful oxidizing and chlorinating agents (Grisham et al, 1984). They differ greatly in antimicrobial activity. Ammonium chloride may be the most potent microbicidal agent produced by PMNs because it is membrane permeable and highly reactive (Thomas et al, 1986).

Oxygen independent antimicrobial mechanisms on the other hand include activities of lysosomal proteases,

other hydrolytic enzymes, and proteins and peptides that bind to microorganisms and disrupt essential processes or structural components (Thomas et al, 1988). If PMNs are successful in engulfing microorganisms they are usually able to eliminate them shortly after the ingestion via oxygen-dependent and -independent killing pathways.

Neutrophil functions

The main functions of polymorphonuclear leukocytes in the host include resistance to infectious diseases or immunity, and production of inflammation, necrosis and fever.

For the first role, clinical observations indicate that neutrophil maintains control of the "frontier outposts" which interface with the microbial population. A steady flow of neutrophils into the areas is necessary to sustain this control although at times the host appears to be able to tolerate prolonged periods of markedly diminished rates in this flow (in neutropenia where blood neutrophil concentration is below the normal range) (Dale et al, 1979; Kyle, 1980). The action of the neutrophil as an essential agency for protection of the host is best evidenced by an enormous increase in susceptibility to sepsis of persons afflicted with agranulocytosis, a clinical condition in which neutrophils are conspicuously absent, and in chronic granulomatous disease (Bodey et al, 1966; Quie et al, 1967; Malech and Gallin, 1987).

Neutrophils also serve some functions in which their phagocytic capacity is not primarily involved. In the inflammatory reaction, neutrophils determine the severity or the production of inflammation itself. The same oxidative and non-oxidative processes that are so important in clearing up microorganisms can act on adjacent tissues and be important in the pathogenesis of a number of non-infectious diseases as reviewed by Malech and Gallin (1987). Antimicrobial agents which are known to be deleterious to neutrophils themselves may leak out of partially closed phagosomes or even be secreted directly into the microenvironment resulting in tissue destruction (Baehner et al, 1977; Weiss, 1989). Pyrogen production is activated by phagocytosing bacteria or after exposure of cells to endotoxins eventually giving rise to fever (Dinarello et al, 1974).

Section 3

Leukocyte locomotion

One of the most striking properties of leukocytes is their ability to crawl about on blood vessels, in tissues and on foreign surfaces. Emigration of leukocytes into inflammatory areas is one of the most fundamental events in pathology. The cells usually begin movement by sending out a new pseudopod. The rate of locomotion varies depending on the physical and chemical nature of the environment. A response to an environmental stimulus takes the form of directed orientation (taxis) or undirected motion (kinesis). Cell locomotion is a complicated process involving coordination of a large number of mechanochemical events.

Cells that leave the blood and accumulate in inflammatory foci must have locomotor capacity. Leukocyte precursors may be devoid of this ability but seem to acquire it during the maturation process (Giordano et al, 1973). Similarly with the neutrophils, their intrinsic locomotor capacity is observed to be acquired during maturation in the bone marrow before leaving for the bloodstream. Fontana and colleagues reported their findings of developing myeloid cells from normal bone marrow acquiring an increased number of

chemotactic factor receptors as they proceed with the maturation event (Fontana et al, 1980). The promyelocyte line HL-60 which is non-motile, acquired locomotor capacity as shown by the increased numbers of chemotactic receptors after being induced to maturation (Niedel et al, 1980).

Random locomotion

This form of motion is random in direction along a path and the axis of the moving cell is not oriented towards a stimulus. If a single leukocyte is tracked, the random walk is not typical Brownian movement but the path is smoothly curving for most of the time (Allan and Wilkinson, 1978). Occasionally the cell rounds up and starts off again in a different direction.

Chemokinesis: A kinesis is a change in intensity of random locomotion. It includes responses to external cues with alterations of speed or frequency of turning but not alterations in direction (Keller et al, 1980). The kinesis response may be of two types with different effects. The first type is called orthokinesis meaning that the speed or frequency of locomotion is determined by the magnitude of stimulus. The reaction is simple as long as the orthokinetic agent is present in isotropic concentration. In an anisotropic environment, orthokinesis can result in cellular accumulation due to slowing down of speed (Wilkinson et al, 1984).

Klinokinesis is a change in the frequency or amount of turning determined by the magnitude of the stimulus. The importance of this reaction is yet to be evaluated but it seems likely that if the cell follows a random path with plenty of turnings, its displacement would be lower than that of cells following more persistent paths (Shields and Haston, 1985). Changes in both speed and in turning are not restricted to chemical stimuli only but may be inducible by the environmental physical properties.

When present at uniform concentration, leukocyte attractants can stimulate locomotion. Leukocytes show a 'persistent random walk' under these conditions (Allan and Wilkinson, 1978). The speed of locomotion is dependent on the concentration of attractant, thus allowing the locomotor reaction to be classified as orthokinesis. Klinokinesis can also be modified by attractants as observed by Keller and co-workers (Keller et al, 1984a). Many physical factors such as temperature and adhesiveness can affect the speed of cell movement without influencing its direction. Other agents are able to alter the cell speed biochemically by-passing the cell surface receptors. Colchicine, a microtubule depolymerizing agent, causes polarisation of a high percentage of leukocytes but is unable to orientate the cell if presented in a gradient (Keller et al, 1984b). As long as there is a uniform concentration of chemotactic agent, accumulation of cell population will not ensue. Cell accumulation can result even in the

absence of chemotactic cues provided that the concentration of chemokinetic agent is anisotropic (Wilkinson et al, 1984).

Directional locomotion

This mode of motion denotes a preference for or avoidance of particular directions. Two responses to environment are known to cause directional locomotion in leukocytes: chemotaxis and contact guidance (Wilkinson, 1985).

Chemotaxis: This is a special form of locomotor response to chemical signals in which the responding cell becomes oriented in, and moves up a concentration gradient of an attractant. In isotropic concentration the same attractant will activate locomotion through similar biochemical pathways but the movement is random in direction (Zigmond, 1974). Leukocytes most likely will not move unless stimulated to do so by an external signal. Blood neutrophils if carefully purified remain spherical. In the presence of a chemotactic factor at an optimal concentration (10^{-8} M FMLP), greater than 95% of cells take up typical polarised locomotor morphology within few minutes (Zigmond et al, 1981; Shields and Haston, 1985). On an appropriate surface, these cells move in the direction determined by the anteroposterior polarity. Polarisation and subsequent locomotion therefore cannot be the responses to a gradient per se.

A probable explanation of the event then is that the cell responds by polarising in the direction of the first hit by ligand on condition that ligand concentration is low enough for cell to differentiate between the first and the subsequent hits. If ligand concentration is isotropic, the first ligand hit on different cells in a population will be random in direction and so will the polarisation reaction. In a gradient however there is a greater chance that the first hit will be on the side of the cell facing the gradient source. As the gradient gets steeper, the

higher the occupancy of receptors by ligand, presenting a higher chance of cells polarising and moving towards the source of gradient. Since the majority of the receptors are now localised at the front portion of the cell, there is an increased chance of further signals being recognized inducing the cell to continually move forward towards the source (Wilkinson and Haston, 1988). Chemotaxis is no doubt an excellent behavioural reaction for cell accumulation providing a likely answer for the rapid influx of neutrophils into the inflammatory lesions.

Contact guidance: A reaction usually defined as a locomotor response in which the direction of locomotion is determined by the shape, arrangement or curvature of the substratum with which contact is being made (Lackie,

1986). The mechanism of this form of directional locomotion is not well understood. It is postulated to be dependent on physical features of the environment without requiring any specialised sensory systems. Cells in aligned collagen gels are constrained to move only in one axis (the axis of alignment of the gel) but free to move in both directions in that axis (Wilkinson et al, 1982). The ability of cells moving through aligned gels to respond to chemotactic gradients is influenced by the gel alignment. Better responses are seen if the axis of fiber alignment is parallel to the axis of the chemotactic field (Wilkinson and Lackie, 1983). This is a simple example of an effect of tissue patterning on the direction of cell locomotion. More complex tissue architecture will definitely impose constraints on cell locomotion in vivo.

Morphology and biochemistry of cell locomotion

Leukocyte crawl by attaching to surfaces and acquiring an asymmetrical shape rather than swimming. Most leukocytes are rounded up with a uniformly ruffled membrane in the absence of an external stimulus. When this uniformly organized membrane is perturbed, protrusion of a hyaline membrane (veil or lamellipodium) can be seen in the direction of locomotion (Ramsey, 1972; Senda et al, 1975). The veil appears to be pushing forward while the remainder of the cell seems to be pulled forward behind the advancing veil. It is in this

region of the veil that the cell is most adherent to the substratum. Thin-section of electron micrographs have revealed the absence of cytoplasmic granules in the hyaline ectoplasm of locomoting leukocytes (Fukushima et al, 1954b). Instead, the principal structure of the veil is made up of microfilaments and changes in their architecture account for the undulatory shape changes of the neutrophils (Oliver et al, 1978). The rear of the moving cell meanwhile acquires a knob-like projection called the uropod or tail where capping takes place (Fukushima et al, 1954a).

A variety of ligands which attach to receptors on the external surface of the cell stimulate these receptors to aggregate to one pole of the cell, a phenomenon known as capping (Oliver and Berlin, 1982).

Motor apparatus

Ultrastructural studies have shown that the pseudopods of migrating neutrophils are rich in microfilaments (Boyles and Bainton, 1979; Fechheimer and Zigmond, 1983). Shape and movement of cells are thought to be mediated by these filaments, composed of the polymerized globular protein, actin found in large quantities in the cytoplasm of leukocytes. Cytochalasin B, a cytoskeletal disrupter was observed to inhibit chemotaxis and diminish the formation of filament-rich pseudopods by preventing the binding of monomeric actin

to the growing end of filaments (Malech et al, 1977; Ryder et al, 1984). In the resting cell, we would expect a dynamic equilibrium to persist between the free and bound forms of monomeric and filamentous actin with the actin-binding proteins. Hence, these morphological changes appear to be tightly coupled to changes in the polymerization state of actin with a change in the manner in which filaments are organized in the cytosol (Schiffmann, 1982). In addition to actin polymerization, chemotactic peptide has been shown to cause phosphorylation of myosin, a muscle fibril protein (Fechheimer and Zigmond, 1983). Calmodulin-dependent protein kinase presumably controls the phosphorylation of the myosin light chain in the presence of micromolar concentration of calcium ions (Fechheimer and Zigmond, 1983). This fact, together with the observation that myosin is present in extending pseudopods (Valerius et al, 1981), is suggestive of contractile forces being exerted in the local environment of the extended pseudopod. This could allow for the pulling of the cell body via the cytoskeleton and membrane-bound actin filaments in the direction of the extending pseudopods (Southwick and Stossel, 1983).

Chemotactic peptides also induce changes in the polymerization of microtubules, proteinaceous organelles present in nearly all eukaryotic cells (Nath and Gallin, 1986). Microtubules are made up of subunits of tubulin

molecules that are capable of influencing cell shape, motility and mitosis. Their role in cell locomotion has been suggested as indirectly evidenced from early studies using microtubule disaggregating agents such as colchicine and vinblastine (Edelson and Fudenberg, 1973; Ramsey and Harris, 1973). Chemotaxis, polarity and integrity of shape of locomoting neutrophils were among those observed to be depressed or abolished. A visual study, however, showed that colchicine-treated neutrophils reacted chemotactically to a gradient source (Allan and Wilkinson, 1978). The cells showed irregular, erratic paths with wide angles of turning, and preserved the usual anterior lamellipodium while locomoting in the up-gradient orientation. Therefore it seems that microtubules are not an obligatory requirement for locomotion but may contribute to the locomotive coordination of these cells. The proposal has been substantiated by a study using anucleate fragments of human PMN lacking microtubules (Malawista and De Boisfleury, 1982). These fragments responded positively to a chemoattractant indicating the lack of a requirement for microtubules for chemotactic locomotion.

Section 4

Methods of studying leukocyte locomotion

Historical background: During the 19th century, many studies were made of leukocyte adhesion to the blood vessels and subsequently emigration into extravascular tissues in inflammation. Many of these reported events were noted following the injections of bacteria or irritant chemicals. In 1887 Hess modified these techniques by introducing anthrax bacilli in a glass chamber which he placed beneath the skin of the tested animal. He then observed various degrees of leukocyte accumulation in the chamber without recognizing the effect being a result of chemotactic migration of the cells. A year later Leber, using a capillary tube technique, inserted capillary tubes containing substances to be tested into the conjunctiva or cornea of rabbit eyes. Bacteria and tissue extracts attracted leukocytes into the tubes, but he was uncertain whether this cellular accumulation was due to random migration or chemical attraction. In answering the question, he proceeded to excise the cornea after injecting the test substances and was able to observe directly active cell migration to the test site continuously. He proposed the theory that chemotaxis was the working force in bringing leukocytes into inflammatory lesion and the foreignness of the test

substance is one of the important requirements in the initiation of cell migration. The importance of Leber's findings was well accepted and recognized firstly by Metchnikoff. With the assistance of his co-worker, [he] confirmed that dead or live bacteria were capable of attracting leukocytes after inserting capillary tubes into the peritoneal cavity (Metchnikoff, 1893).

Between 1890 and 1900, there were many reports with similar results employing the same technique of injecting test substances into tissues of animals or using capillary tubes (reviewed by McCutcheon, 1946; Harris, 1954). Several workers tried to replace the techniques but were unsuccessful. Clark (Clark and Clark, 1920; Clark et al, 1936) attempted a different approach by introducing test substances into transparent tadpole tails and observing subsequent leukocyte migration from the blood vessels through the tissues towards the injected materials. The method was consequently widely and successfully adapted in studying inflammatory responses even though it was technically difficult. Great care is required since any slight trauma is likely to excite leukocyte emigration from the vessels. A reliable in vitro method in demonstrating chemotaxis was unobtainable until the work of Commandon in 1919 (Wilkinson, 1982). He was the first to observe leukocytes moving towards red cells infected with parasites using time-lapse cinematography. McCutcheon

and his colleagues (McCutcheon et al, 1934; Dixon and McCutcheon, 1936) improved the slide and coverslip method of Commandon to obtain some basic quantitative data on leukocyte migration with the help of a camera lucida. Comparison between random and chemotactic migration was possible following their technique. Cells were found to respond to a chemotactic gradient at a distance up to a millimeter but fewer cells responded as the distance increased. Time-lapse cinematography was useful in Lewis's studies on leukocyte locomotion detailing the behaviour of cells in the absence of a chemotactic gradient with sequential tracings of changes in cell shape (Lewis, 1934). Using rat neutrophils, he was the first to describe the typical locomotor morphology of these cells. Constriction rings at both anterior and posterior ends which separated the pseudopodal part from the tail were observed. While most of the studies were conducted on laboratory animals, Rebuck and Crowley devised a technique known as the skin window on human skin (Rebuck and Crowley, 1955). A lesion was made on the forearm by scraping with a scalpel or abrasion with high speed drill before applying test substances to the area. The test site was covered with a sterile glass coverslip on which cells migrating from the dermis will adhere. The coverslip was stained and the cells counted. ~~Counted~~ In 1962, Boyden developed another new technique for the measuring

of leukocyte chemotaxis. Substances were separated from cells by micropore filters with a pore size that enabled the cells to squeeze through the filter during active migration. Cells were allowed to settle on top of the filter and chemotactic factor was placed underneath allowing it to diffuse through to the top hence setting up a gradient (Boyden, 1962). His invention inaugurated a new era in studying leukocyte locomotion in vitro.

Recent techniques

Cell locomotion is a complex phenomenon very much affected by the environmental changes. To assay the phenomenon accurately is not a straightforward task with different assays available useful in different contexts. Full information about a particular aspect of cell movement may be obtained only by employing more than one assay. Methodologies for studying locomotion can be classified into two major groups. The first group includes those assays in which a large population of cells is allowed to move in the presence or absence of locomotor stimulants. After a time interval the locomotion is stopped to examine the distribution of the cell population. Included in these widely used endpoint assays are the agarose and micropore filter assays. The second group focuses on analysing the locomotory behaviour of each individual cell visually. Time-lapse cinematography, orientation assay, polarisation assay,

and collagen or fibrin invasion assay are the methods offered within this category. Detailed behavioural studies of the cells can be accomplished throughout the whole experimental time period with this batch of assays.

Micropore filter assay: This simple assay initially introduced by Boyden is still among the most popular assays available (Boyden, 1962). A porous filter with pores of a size sufficient to permit cells to crawl through actively, but not to fall through passively, is used to separate two compartments. Cells are placed in the upper compartment while the attractant is in the lower region. Molecules of the attractant will diffuse through pores of the filter forming a gradient, thus activating the cells to respond by migrating through the partition downwardly. After the appropriate time the filter is fixed and stained. Three possible ways can be applied in evaluating the locomotion of these cells.

The first choice is to determine the distance migrated by the leading front cells. This method was first used by Weksler and Hill (Weksler and Hill, 1969) and later by Zigmond and Hirsch (Zigmond and Hirsch, 1973). The cells are incubated for a length of time such that the fastest moving cells have moved to a reasonable distance through the filter without going over to the lower surface. After fixation and staining, the filters are mounted topside up

and observed under a microscope. The micrometer fine-focus adjustment is racked down past the leading front of cells. It is then racked back up to the leading plane where the nuclei of two or more cells are visibly together in the same field. A micrometer reading is taken at this position before the fine adjustment is ~~racked~~ racked up to the top surface of the filter to record a second reading. The difference between the two readings gives a measure of the distance migrated by the leading front of the cells. At least five readings are taken at different fields from each filter. This is quite an accurate and reproducible method as it is less dependent on the initial cell population placed on the filter. Another advantage of using this option is that it can be used for 'chequerboard' studies (Zigmond and Hirsch, 1973). In this study a series of chambers is set up with the cells exposed to a range of positive, negative, and zero gradients. An orthokinetic dose-response curve is given in a series of tests with varying absolute concentrations but in the absence of a gradient. If cells in positive gradients show greater, displacements than would be expected from observed ~~displacements~~ displacements in the absence of a gradient, this gives ~~indirect evidence for chemotaxis.~~ Disadvantages of the method include its relative insensitivity to changes in the proportion of cells in any population that are able to translocate. A major drawback of the assay is that it

does not allow direct visual observation of the paths of the moving cells. The second method to evaluate cell locomotion is by determining the number of cells which have reached a given point. The oldest and easiest version of this method is to count the cells on the lower surface of the filter even though it is inaccurate because cells that have passed the lower surface may have dropped off. To counteract such a problem, an alternative procedure has been recommended involving the use of a second filter to trap the non-adherent cells (Keller et al, 1972). Counting of cells can also be done at a chosen plane within the filter and beyond it. This gives better results than the former way of counting cells because it eliminates cell loss due to detachment.

Determining the total number of cells per field entering the filter and their distribution in the filter forms the basis of the last method in assessing the cell movement. The procedure provides information about all cells that have penetrated the filter in a field and how they are distributed. The approach is time consuming and only practicable with an automated counting machine (Van Dyke et al, 1979).

Agarose method: Nelson and co-workers were the first to describe chemotaxis of human neutrophils under agarose (Nelson et al, 1975). The fundamental principle

of the assay is similar to that of immunodiffusion tests in which three circular wells are bored into agarose on a glass or plastic slide. Chemoattractant and control solution are placed in the two outer wells while the center well is filled with cell suspension. With time the chemotactic factor diffuses radially through the gel attracting the cells from the central well. In response the cells crawl within the space between the agarose and the slide. As a result of the activity an egg-shaped pattern of cell distribution is established, with the pointed end facing the well containing the attractant formed. Essentially this method measures the locomotion of a cell population but has the advantage of being used as a visual assay, permitting motile behaviour of a single cell to be studied.

Visual assays

Additional information about cell locomotory behaviour can be obtained by direct observation of locomoting leukocytes. Nevertheless, the assay requires more sophisticated and expensive apparatus and is time consuming if detailed analysis is required.

Polarisation assay: The assay, sometimes known as the shape change assay, is a simple but important new assay based upon the change in shape of cells in suspension when stimulated by an agonist. An unstimulated neutrophil if carefully isolated exhibits spherical

morphology without morphological polarity, but adopts a non-spherical or polarised morphology in the presence of a stimulus (Haston and Shields, 1985). Various parameters such as time course of polarisation, degree of polarisation, and proportion of polarised cells can all be measured. Even though the assay fails to provide further information beyond the initial locomotor events, a large number of potential factors can be screened for any stimulating activity. Another advantage of the assay is that it offers rapid assessment of the immediate locomotor response to both agonists and antagonists.

Chemotaxis assay of cell locomotion. One disadvantage of the assay is that certain compounds such as colchicine, a microtubule-disrupting agent, induce polarisation but they are not classified as chemoattractants (Keller et al, 1984b). Generally factors which induce chemotactic responses in micropore filters are found to stimulate significant polarisations in suspension.

Orientation assay: The assay, making use of a chamber, is a convenient way of studying the neutrophil's ability to perceive a gradient (Zigmond, 1977). No translocation on the part of the cells is needed in demonstrating their recognition of the attractant source except to orient themselves to the gradient. The chamber is a perspex slide cut to form two grooves separated by a narrow bridge. These grooves can be

filled with different concentrations of chemoattractants or one is filled with a stimulant and the other with a control solution. Cells are allowed to adhere to a coverslip which is then inverted over the slide secured by two brass spring-clips. Morphological orientation of the cells in the gradient is observed hence providing a rapid means of identifying if any given attractant is a true chemotactic factor.

Time-lapse cinematography: To analyse the movement of cells observed microscopically a filmed record is essential. It is impossible to track the motion of a cell population by eye in a single field for a long period of time considering the usual slow speed of a moving neutrophil, 20 micrometer per minute. Time-lapse cinematography can facilitate the task by using a lapse interval that allows a film to show events happening faster than they did in actual time. Cinefilm and videotape are two recording media available at present with the former being superior for high-power filming of detail changes of cell in motion (Haston and Shields, 1984). Videotape has the advantage of being able to play back immediately but with poorer images. A microscope with adequate optics and a heated stage of 37° C with a cinecamera should equip the basic requirements for the assay. Behavioural analysis of the cell population is done using phase-contrast optics at low magnifications because a large field with enough cells is needed for

statistical purposes. Higher magnifications with contrast optics are used to study detailed shape changes of a single cell. At the beginning, analysing cell tracks was done by projecting the film onto a drawing paper (Allan and Wilkinson, 1978; Shields and Haston, 1985), but recently, a microcomputer has been used to automate the technique (Dow et al, 1987).

Visual 2-D: It is possible to gain information on behavioural responses of neutrophils to chemotactic factors aided by time-lapse cinematography on 2-dimensional substrata such as serum or albumin coated glass or tissue culture plates (Allan and Wilkinson, 1978). The substratum is coated with protein, which should not act as a stimulant, to reduce adhesiveness thus allowing the neutrophils to locomote on the 2-D plane. The assay has been shown to be helpful in obtaining data on the effects of agents like colchicine that tend to alter chemotactic behaviour of cells towards an attractant.

Visual 3-D: The use of collagen and fibrin gels in studies of cell locomotion is becoming increasingly popular since they simulate a physiological matrix. In response to the ~~chemical~~ attractant, the leukocytes will migrate into the transparent 3-dimensional fibrous matrix of the gel, and their paths can be followed (Shields et al, 1984). Type 1

collagen which can be prepared from rat tail tendon (Elsdale and Bard, 1972) forms a gel within few minutes of being restored to physiological pH and ionic strength. Fibrin gels are prepared from thrombin and fibrinogen. Chemoattractants are usually incorporated into or layered on top of the gels in forming the desired gradients before adding the cell suspension on the surface. An appropriate time period is allocated for the cells to invade the gel matrix successfully (Islam et al, 1985). Gel invasion assays can be adapted for assessing net population movement either by the leading front method or counting the cells at measured distances through the gel after fixing the gel with glutaraldehyde. Gel assays can also be used to study contact guidance. There are, however, technical problems to be encountered when employing protein gels. It is quite difficult to manipulate them as they are fragile, and counting of cells is not easy if there is any vibration. Sometimes cells fail to invade the gel even though locomotor morphology has been achieved.

Section 5

Chemiluminescence

Chemiluminescence is the production of light by chemical reactions involving the formation of electronically excited states.

The first report of chemiluminescence was made by Albrecht in 1928 (Stross and Branch, 1938) who was described to have observed bright chemiluminescence when luminol, a cyclic hydrazide, was oxidised. Oxidation of luminol can be carried out in protic media such as water or in aprotic solvent such as dimethyl sulfoxide. The phthalate ion produced almost quantitatively during luminol oxidation is found in an electronically excited state and is responsible for the observed emission.

Chemiluminescence by phagocytic cells: The generation of chemically reactive molecules as a result of the respiratory burst in PMN, is an essential step in host defense against microbial invaders. Chemiluminescence of PMNL is a phenomenon related to the activation of the respiratory burst as originally reported by a group of investigators demonstrating light emission at the onset of phagocytosis (Allen et al, 1972; Cheson et al, 1976). The response is influenced by a number of factors such as temperature, ions and media (Andersen and Amirault, 1979) and can be amplified by the addition of 5-amino-2,3-dihydro-1,4-phthalazinedione or luminol (Allen and

Loose, 1976). Before the introduction of luminol, the mechanism of light emission from the phagocytes was shown to involve both superoxide anion (O_2^-) and the enzyme myeloperoxidase (Rosen and Klebanoff, 1976). As a result of adding luminol chemiluminescence production was found to be totally dependent upon the MPO- H_2O_2 system (DeChatelet et al, 1982). Their studies showed that the presence of MPO in extracellular fluid is insufficient to obtain a normal chemiluminescence response from MPO-deficient PMNL indicative of the light emission reaction taking place both intra- and extracellularly. In luminol-enhanced chemiluminescence systems, the reaction may be limited by the diffusion of luminol into the cells and not by the generation of oxidative metabolite.

Section 6

Adhesion membrane receptors

Interaction between granulocytes and other cells or extracellular matrix is of central importance in a wide spectrum of granulocytic functions which contribute to host defense against foreign infections. Mobilisation of these cells in vivo is influenced by the nature of these interactions (Atherton and Born, 1972). Adhesive interactions of granulocytes with vascular endothelium or recognition of opsonised particles have been shown to be facilitated by membrane receptors. There is increasing evidence that the Mac-1, LFA-1 family of glycoproteins also named the CD11/CD18 family of adhesion-promoting receptors are of general importance in the aforementioned granulocyte functions (Springer et al, 1984). Recently leukocytes were discovered to express a family of cell-surface glycoproteins, CD11a/CD18 (LFA-1), CD11b/CD18 (CR3), and CD11c/CD18 (p150,95), that mediates the interaction of phagocytes with microbes opsonised with complement components C3bi (Graham et al, 1989), and adherence of leukocytes to vascular endothelium (Luscinskas et al, 1989). The molecules appear either to function independently or synergize with other receptors in regulating a range of functional interactions.

Study of leukocyte adhesion deficiency

In early 1979 a group of patients manifesting widespread bacterial infections, defective neutrophil mobility and delayed separation of the umbilical cord was identified (Abramson et al, 1981). A year later it was proposed that the defective neutrophil functions in those patients were secondary to an abnormality in adhesion (Crowley et al, 1980). Subsequently other investigators reported similar findings of patients with extreme leukocytosis but who failed to form pus at sites of infection. This is suggestive of the failure of leukocytes to extravasate and their retention in the vasculature, due to their inability to adhere to endothelial cells as observed in vitro.

Characterisation of the proteins missing from patients' cells have confirmed their role in all adhesion related events. With the advent of specific monoclonal antibodies for the three alpha subunits (CD11a,b,c) and the common beta subunit CD18, researchers were able to identify patients having congenital deficiency in all three alpha-beta complexes on their leukocytes (Springer et al, 1984). Patient granulocytes are deficient in both the intracellular stored pools and surface expression of the complexes. The actual genetic defect of this inherited disease is in the gene for CD18 or beta chain whereby precursors for the subunits are found to be

abnormal though they have normal alpha chains (Kishimoto et al, 1987a).

Almost all patients studied have been observed to manifest profound abnormalities of neutrophil adherence to substrates and adhesion-dependent functions including chemotaxis, orientation and aggregation. The respiratory burst fails to be triggered due to poor phagocytosis of opsonized particles and antibody-dependent cellular cytotoxicity is also noted to be abnormal. On the other hand adherence-independent cellular functions like cell polarisation, receptor binding of FMLP, and intracellular microbicidal activity are reported to be relatively normal reflecting the presence of normal IgG, Fc, CR1 and other receptors on CD11/CD18 deficient granulocytes (Springer and Anderson, 1986).

Structure of glycoprotein family

The distribution of the CD11/CD18 family of trans-membrane glycoproteins is limited to white blood cells and haematopoietic precursor cells (Kurzinger et al, 1981; Krensky et al, 1983; Miller et al, 1986). The members of the family each comprise non-covalently associated alpha and beta polypeptide chains with the absence of disulfide bonds (Kurzinger and Springer, 1982; Sanchez-Madrid et al, 1983). The polypeptide complexes are not only present on the cell surface but also in intracellular vesicles. Alpha chains of this subset of the

_____ integrin superfamily have molecular weights of 195 kD (CD11a), 185 kD (CD11b), and 150 kD (CD11c) and they are homologous to each other. The beta chain (CD18) is a 95 kD polypeptide bearing no resemblance to the alpha chain. and is identical for all the three proteins.

Molecular studies of both chains reveal extensive homology to other integrin receptors thus confirming their memberships in the supergene family of integrins (Hynes, 1987). The glycoproteins require divalent cations to stabilize interactions between the two subunits along with warm temperature and an intact microfilamentous cytoskeleton for their functional abilities.

Biosynthesis

Biosynthesis of the molecules has been studied in humans and mouse with the following model emerging (Ho and Springer, 1983; Miller and Springer, 1987a). In normal cells both subunit precursors are synthesized and become non-covalently associated, most probably in the endoplasmic reticulum, before being transported to the Golgi where carbohydrate processing with a slight increase in molecular weight occurs. The mature molecules are then relayed to the cell surface or to intracellular storage sites (Todd et al, 1984; Bainton et al, 1987; Miller et al, 1987b). Inflammatory mediators including C5a and FMLP stimulate a 5- to 10-

fold increase in expression of Mac-1 and p150,95, but not of LFA-1, on the cell surface (Springer et al, 1984; Anderson et al.1985). Increased surface expression is not impeded by protein synthesis inhibitors. Upregulation is apparently of great importance in regulating leukocyte adhesiveness and mobilization of the molecules from the latent storage pool to the cell surface can be readily switched on by inflammatory stimuli.

Characteristics of individual proteins in the family

The first molecule found to be deficient from leukocyte adhesion deficiency patients was CD11b/CD18 (CR3, Mac 1, Mo 1, C3bi receptor) that functions as an opsonic receptor and promotes the binding of iC3b-coated particles which is followed by phagocytosis of the entity (Beeler et al, 1982; Wright et al, 1983). The receptor recognizes surface-bound iC3b but not the precursor C3b or the product of cleavage C3dg. It is composed of two non-covalently linked polypeptides which are exposed to the cell surface and requires high concentration of divalent cations in order to interact effectively with the ligand (Wright and Silverstein, 1982; Wright and Jong, 1986). The binding capacity is known to be temperature dependent. Polypeptide ligands harbouring the triplet Arg-Gly-Asp (RGD) serve as the site of recognition for the molecule and also for many receptors involved in cell adhesion events (Wright et

al, 1987; D'Souza et al, 1988). Recent work has identified additional ligands for this receptor including the binding of protein gp63 on the surface of *Leishmania* and promoting the internalization of the intracellular parasite (Russell and Wright, 1988). Surfaces coated with fibrinogen can also be recognized by CR3 thus promoting adherence of neutrophils to clots and subsequent digestion of fibrin (Wright et al, 1988). Finally the receptor has been shown to recognize an unidentified polypeptide on the surface of unstimulated human umbilical vein endothelial cells (Harlan et al, 1987).

Monoclonal antibodies against the second receptor of the family were used to block T-cell mediated cytotoxicity. Studies have identified 3 'lymphocyte function- associated antigens', LFA-1, LFA-2 and LFA-3, which are needed for efficient cytotoxicity (Sanchez-Madrid et al, 1982). Anti-LFA-1 (anti-CD11a/CD18) antibodies seem to inhibit cytotoxicity by binding to LFA-1 molecules on the killer cell thus blocking adhesion of the cell to target. LFA-1 does not provide specificity for cytotoxic cells but instead functions to strengthen adhesion between target and killer cell (Martz, 1986).

It also participates in adhesion events unrelated to cytotoxicity such as strengthening the adhesion of T lymphocytes to dendritic cells that appears to be a prerequisite for stimulation of resting T cells (Inaba

and Steinman, 1987). The ligand that LFA-1 recognizes on target cells eluded investigators until Marlin and Springer identified it as intercellular adhesion molecule-1 or ICAM-1 (Marlin and Springer, 1987), a surface glycoprotein of molecular weight 90 kDa.

The purified molecule of ICAM-1 inserted into artificial membranes binds cells bearing LFA-1 but not those without the receptors. Structure determinations of ICAM-1 has shown that it belongs to the immunoglobulin superfamily displaying related sequence of Arg-Gly-Glu instead of the usual RGD sequence (Simmons et al, 1988; Staunton et al, 1988). ICAM-1 serves as the ligand for CD11a/CD18 on endothelium since leukocytes adhere more strongly to stimulated endothelial cells that express the molecule on their surfaces.

The third member of the family, CD11c/CD18 or p150,95, is expressed on phagocytic cells, NK cells and certain cytotoxic T cells having a 95 kDa beta-chain structurally identical to the beta-chains of the first two molecules (Lanier et al, 1985; Springer et al, 1986). Unlike CR3 and LFA-1, functions of CD11c/CD18 are just beginning to emerge. Examination of CD18-deficient neutrophils or normal neutrophils treated with specific antibodies for the individual chains suggests that both CR3 and p150,95 have common function in mediating neutrophil adherence to endothelial cells (Miller et al, 1987a). Another study showed that membrane surface

CD11c/CD18 too exhibited iC3b-receptor activity and is probably similar to the neutrophil receptor for iC3b (Myones et al, 1988).

Additional ligands for CD11/CD18

Besides the above-mentioned ligands, CD11/CD18 molecules have been discovered to play a role in recognizing other vital substrates thus revealing their wide range of functional characteristics.

Recognition of microbes in the absence of complement:

Antibodies against CR3 partially inhibited the binding of particles such as zymosan (Ross et al, 1985a), *Staphylococcus* (Ross et al, 1985b), and *Leishmania* (Blackwell et al, 1985) on to phagocytic cells in the absence of complement. From the binding experiments performed, it was proposed that CR3 interacts directly with the particles without involving C3bi. This idea is supported from the observations of leukocyte adhesion deficiency (LAD) patients cells exhibiting defective recognition of both zymosan and *E.coli* in the absence of serum opsonins. Detailed experiments have also indicated that each member of the family shares the capacity to bind to *E. coli* (Wright and Jong, 1986) and *Histoplasma* (Bullock and Wright, 1987). In the experiments, receptor sites for all 3 glycoproteins were cleared using monoclonal antibodies thereby inhibiting the binding of microbes. Binding is still observed if any one of the

receptors is present indicating the capability of individual receptors to bind to the organism separately. The ability of phagocytes to recognize a carbohydrate structure on the microbes and ingest them without opsonization may serve to limit the multiplication of these organisms in early stages of infection before the onset of adaptive immunity.

Recognition of bacterial lipopolysaccharides:

Macrophages bind *E.coli* by recognizing the most prevalent molecule on the surface of the bacterium, LPS,

and two distinct binding sites for LPS on the CR3 molecule

have been identified (Wright and Jong, 1986). This claim has been recently substantiated by Wright and his co-workers when they blocked the binding of C3bi with anti-CR3 but not that of LPS (Wright et al, 1989). Although binding sites for LPS and C3bi are separate, they seem to be functionally linked. The binding site for LPS on the CD18 family appears to be less affected by the identity of the alpha chain since all 3 members exhibit equivalent binding capabilities to LPS. Even though precise structural features of the LPS binding site are yet to be defined, it is agreed that members of CD18 family of receptors on PMN are required for recognition of bacterial LPS. The presence of dual binding sites may be a common property of receptors related to the CD18 family.

Adhesion of leukocytes to endothelium: Increased adherence of circulating neutrophils to the endothelial cell lining, of the microvasculature is an essential step in the early events of inflammation, and precedes neutrophil migration through the vessel wall towards the site of injury (Movat, 1985). For nearly a century, chemotactic factors have been regarded as key participants in the process of leukocyte diapedesis demonstrated both in vivo and in vitro. With the discovery of leukocyte surface adhesive glycoproteins, the mechanism of neutrophil adherence has been better understood (Tonnesen, 1989). Documented observations on the adhesive interaction between endothelial cells and leukocytes may indeed be directly induced by physiologically relevant chemotactic stimuli. The major subject though is the confirmation of the key role played by the glycoprotein family in this interactive process. In the study conducted by Tonnesen (1989), leukocytes of patients with LAD showed markedly diminished adherence to endothelial cells in response to chemotactic stimulation so did normal cells challenged with MAbs specific for beta subunit. Margination, thought to be a reversible spontaneous interaction of unstimulated neutrophils with vascular endothelium, seems to occur independently of the receptor family as indicated by the non-inhibitory effect of antibodies on non-stimulated adherence of normal cells. Surface expression of the adhesive glycoproteins is

obviously essential for neutrophils in enhancing adherence to the endothelium before diapedesis in the presence of a variety of inflammatory mediators (Smith et al, 1988). In addition to the expression of CD11/CD18 complex of glycoproteins for localised adhesion of blood leukocytes to vessel walls at sites of inflammation, a contribution of endothelial-leukocyte adhesion molecule-1 (ELAM-1) to the mechanism of leukocyte adhesion has been reported (Bevilacqua et al, 1987b; Luscinkas et al, 1989). Results from these in vitro studies suggest that PMN adhesion to activated endothelial cells involves two separate cellular mechanisms: a leukocyte-dependent mechanism involving CD11/CD18 and an endothelial-dependent mechanism involving ELAM-1.

Regulation

The act of mediating adhesive interactions by the CD11/CD18 family is under strict physiological control. Normal circulating neutrophils do not stick to the vascular endothelium but tend to do so with dependence on the receptors after challenging with C5a. An example of regulation involves the binding of C3bi-coated particles to CD11b/CD18 on neutrophils after introducing a stimulus that will induce a 2-to-14 fold increase in expression of this glycoprotein family on the cell surface (Wright and Meyer, 1986). Current evidence indicates the resulting upregulation is due to mobilization

of preformed molecules from the intracellular storage sites perhaps the specific granules and/or the gelatinase-containing organelles to the plasma membrane (Petrequin et al, 1987; Bainton et al, 1987). At the same time evidence is slowly accumulating which throws doubt upon the concept of stimulus-induced upregulation and increased surface expression are required for the enhanced adherence. There is a proposal that subsequent biochemical or conformational modifications, an interaction with another membrane component or possibly an alteration of density distribution of glycoproteins within the membrane may be required to produce the actual adhesive site once the molecules have been projected on to the cell surface (Detmers et al, 1987).

Section 7

Lipopolysaccharides

Lipopolysaccharide is a macromolecular substance firmly bound to the surface of gram-negative bacteria forming an integral part of the outer membrane unit (Inouye, 1979). The molecule seems to elicit a variety of the clinical manifestations of the diseases caused by gram-negative bacteria in humans (Emond et al, 1978). Unlike many bacterial exotoxins, LPS does not have enzymatic activity that disrupts normal host cell physiology. Rather LPS is thought to activate host cells to release abnormal amounts of endogenous mediators such as IL-1 and TNF which are able to alter physiologic homeostasis in ways that may be toxic or beneficial to the infected animal (Beutler et al, 1985; Durum et al, 1985).

A distinction between endotoxin and lipopolysaccharide is needed to understand the role of each molecule in the context of causing diseases. Endotoxin consists of an outer membrane macromolecular complex of lipid, protein, and polysaccharide (Hitchcock et al, 1986). LPS is a molecule of covalently bound lipid and carbohydrate separated from the bacterial cell wall and from protein and other constituents (Westphal et al, 1983). LPS possesses all the biological activities of endotoxin.

Structure

Chemically LPS consists of 2 parts of contrasting chemical and physical properties: a hydrophilic polysaccharide and a hydrophobic lipid, lipid A (Orskov et al, 1977). The polysaccharide is further subdivided into the O-specific polysaccharide and the core oligosaccharide. The former is built up of repeating oligosaccharide units whose structure and composition vary for different LPS while the latter is more conservative in its structural make-up (Nghiem et al, 1982). Hydrophobic lipid A is the most highly conserved part of LPS being similar among the gram-negative bacteria.

For structural and biological investigations, mutant strains, the so-called R (rough) mutants have been of great value. These mutants have a genetic defect in the biosynthesis of complete LPS lacking in O chains or O chains and part of the core (Mäkelä and Stocker, 1984). The most defective types of LPS which are synthesized by Re mutants (sometimes referred as deep rough or heptoseless LPS) only contain KDO (unique 2-keto 3-deoxy-octulosonate) linked to lipid A. Apparently Re LPS is the minimum requirement to maintain integrity of the gram-negative outer membrane. LPS with intact O-antigen are denoted as smooth form.

Isolation and purity

When studying the biological properties of LPS purity is an important prerequisite since the presence of other bacterial components will affect these properties (Hitchcock and Morrison, 1984). Nucleic acid, proteins, phospholipids and glycans are the commonest contaminants in LPS. The most widely applied method of isolating LPS has been the phenol-water procedure (Westphal and Jann, 1965). For many years this method was employed to isolate the S- and R-forms of LPS; comparing with other techniques these extracts were characterized by the lowest degree of contaminants. Although relatively pure, these isolates did contain a low but significant quantity of contaminants (1-3% protein) whose complete removal proved enormously difficult suggestive of strong linkage with LPS (Galanos et al, 1969). With the advance of isolation and purification methodology, a considerable improvement in the purity of LPS has been achieved. The introduction of phenol-chloroform-petroleum ether (PCP) extraction procedures made the isolation of R-form LPS possible which is uncommonly free of the commonest contaminants reducing them to less than 0.1%. Hence the term LPS should be reserved for purified bacterial extracts which are reasonably free of detectable contaminants particularly protein.

Solubility in water

The presence of hydrophilic polysaccharides and hydrophobic lipid A endows LPS molecule with amphipathic properties. Solubility of LPS in water depends on the relative proportion of these two regions; increasing with higher proportion of hydrophilic region (Galanos et al, 1977). S-forms, rich in polysaccharides, exhibit greater solubility than the R-forms. Within the R-forms, solubility decreases going from Ra to Re indicating that the number of core sugars parallels a corresponding decrease in solubility. LPS does often show great differences in solubility which is unexplained by the polysaccharide contents. R-form preparations isolated by the PCP method are generally more soluble than the corresponding phenol-water extracts, even in some cases better than phenol-water extracts of S-form. The presence of negatively charged phosphate and carboxyl groups plays an important role in solubility properties of LPS especially for the R-form and free lipid A. Divalent cations (Mg^{2+} and Ca^{2+}), needed to neutralize these negatively charged groups, have a marked effect on the solubility of R-forms causing them to aggregate and precipitate from aqueous solutions.

Relationship of LPS structure to biological activity

There has been an appreciation that discrete structural components of the endotoxin molecule are

responsible for the biologic activity. Studies employing endotoxins from *Salmonella* mutant strains provided evidence that LPS is responsible for many of the biological properties of endotoxins (Kim and Watson, 1967). Furthermore the lipid A moiety of LPS plays a prominent role in the expression of endotoxicity, as demonstrated in studies using LPS derived from core-defective mutants and free lipid A. In addition to using endotoxins from bacterial mutants to establish structure-function relationships, selected portions of the molecule modified by physicochemical procedures were also utilized (Neter et al, 1956). Subsequently the studies involved the use of the cationic antibiotic polymyxin B to modify LPS activity through stoichiometric binding to the lipid A region (Morrison and Jacobs, 1976). Procedures to modify LPS structural chemistry have contributed significantly to the knowledge of LPS biological activities in relation to its structure.

Interaction with host mediation systems

Bacterial endotoxins exert an impressive array of biological effects on the susceptible host by virtue of their capacity to interact with a number of mediation systems of the host (reviewed by Morrison and Ulevitch, 1978). The precise role in mediating certain pathophysiologic events associated with gram-negative

sepsis in humans remains unresolved. Immune responses to endotoxins may occur via the humoral system resulting in the production of specific antibody. Such antibody could promote clearance of the endotoxin by the reticuloendothelial system or sensitize an endotoxin-containing microbe to complement mediated lysis or phagocytosis. Alternatively a cellular mechanism may be involved, with the production of activated cells that would aid in eliminating the microorganisms.

Effects of endotoxins on neutrophils

Neutrophils represent an important target for bacterial endotoxins. In addition to their ability to alter blood levels of circulating neutrophils (as reflected in neutropenia or leukocytosis), bacterial endotoxins are capable of influencing the locomotory, metabolic and bactericidal properties of neutrophils. Endotoxin-neutrophil interactions appear to be highly complex with both direct and indirect consequences. Studies demonstrating that a concentration of endotoxin of less than 1 microgram per milliliter is sufficient to modify neutrophil functions are consistent with the hypothesis that endotoxin may mediate at least some of the effects on neutrophil behaviour observed in septic patients (Dahinden et al, 1983; Haslett et al, 1985).

Significant variations among LPS chemotypes cause important variations in neutrophil functions (Pugliese et al, 1988).

Endotoxin binding to the neutrophil: An initial binding interaction is required between the endotoxin molecule and the leukocyte membrane for a direct alteration of neutrophil functions. Only a few studies have addressed the nature of this interaction in a detailed manner either in respect to the receptors involved, the number of binding sites or the mechanism of binding. In 1955 Braude et al observed that the initial rapid clearance of labelled endotoxin injected intravenously into rabbits was accompanied by marked leukopenia. Their work was later pursued by other investigators attempting to evaluate the binding capacity of neutrophils to endotoxins but with complications emanating from factors that might influence the interaction such as the natural form in which endotoxin is presented to the cells. In late 1988 some light was shed on the endotoxin receptors on the cell surface of neutrophils (Wright et al, 1989). Members of the CD18 adhesion family of receptors were discovered to be essential for recognition of bacterial LPS and eventually binding on to the phagocytes.

Effects on adherence and aggregation: Intravenous administration of endotoxin produces an early and pronounced decline in the count of peripheral blood neutrophils because of a drift of these cells to the marginal pool and eventual sequestration in capillary beds. During this period significant changes in the adhesive and chemotactic properties of peripheral blood

neutrophils have been observed. There is evidence, suggesting that certain inflammatory mediators (LTB_4 , C5a , and FMLP) induce increased neutrophil adherence to endothelium via an effect on neutrophils (Tonnesen et al. 1984). Other authors indicate that mediators like LPS may act to enhance neutrophil adhesion on the endothelium either directly or indirectly after exposing LPS to both neutrophils and endothelial cells (Pohlman et al, 1986; Schleimer and Rutledge, 1986; Thomas et al, 1988). Besides stimulating adhesiveness, aggregation of neutrophils in vivo was also reported after injecting bacterial endotoxins into rabbits (Berthrong and Cluff, 1953; Goodman et al, 1979). Induction of cellular aggregation in vivo occurs via an indirect mechanism possibly complement dependent.

Effects on neutrophil motility: LPS elicits a marked acute inflammatory reaction characterized by intense neutrophil infiltration when localised within tissues. It appears to be the primary mediator of neutrophil migration elicited by gram-negative bacteria since treatment with agents that inactivate LPS diminishes this response significantly (Issekutz and Bhimji, 1982). Even though LPS is not a chemoattractant it is a potent inducer of neutrophil infiltration with a minute amount as low as 10^{-3}M sufficient enough to elicit a detectable response (Colditz and Movat, 1984). Following this study a project was embarked upon to determine if any release

of cytokines was involved in the activity of LPS (Cybulsky et al, 1988). To answer the question, they applied tachyphylaxis experiments, local desensitization of tissues to an inflammatory agent, to establish a potential role for cytokines generated in situ by LPS. Significant heterologous tachyphylaxis was observed between IL-1 and LPS implicating the potential role of IL-1 as a mediator of LPS-induced neutrophil emigration. Chemotactic responsiveness was also studied and found to be diminished after exposure to bacterial endotoxins (Haslett et al, 1985). The reduction in neutrophil directed and random locomotion has led to a proposal that endotoxins are capable of "cross-deactivating" these cells to multiple chemotaxins by rendering them hyperadhesive (Dahinden et al, 1983).

Effects on lysosomal degranulation:

Extracellular release of enzymes from neutrophil storage granules can be induced by inflammatory stimuli or trace concentrations of LPS (Haslett et al, 1985). Recently it has been observed that LPS enhanced FMLP-induced neutrophil secretion of elastase as well as MPO and vitamin B₁₂-binding protein (Fittschen et al, 1988). LPS alone does not result in release of LTB₄ by neutrophils but priming them with picogram quantities of LPS increases the production and release of LTB₄ in the presence of a second stimulus (Doerfler et al, 1989). The results thus far suggest that LPS might affect host

defense and tissue injury by potentiating effects of other stimulants on neutrophil release of granule components.

Effects on oxidative and bactericidal capacity: Several investigators have evaluated the potential influence of endotoxin on neutrophil bactericidal activity in general and on oxidative capacity in particular. Guthrie et al (1984) examined the effect of bacterial endotoxin in modifying the oxidative metabolic response and found that LPS did not appear to act as a stimulus in releasing O_2 nor did its presence increase the release in combination with a primary stimulus (Guthrie et al, 1984). However, pre-incubation of the cells with LPS resulted in a significance enhancement of the O_2 release; the priming effect was obtained in the absence of serum in order to study the direct interaction between LPS and neutrophils. Cohn and Morse were among the first to assess bactericidal capacity of neutrophils in the presence of endotoxins (Cohn and Morse, 1960). Enhanced killing was noted when fresh serum was added but no appreciable killing occurred without the serum, an indication of some serum constituents that mediate the phagocytic ability of these cells. Proctor on the other hand observed a depression in subsequent killing of bacteria after pre-exposing the cells to endotoxins (Proctor, 1979). Oxidative metabolic activity of neutrophils was indirectly promoted but subsequent

capacity to mount an oxidative response following challenge with opsonized bacteria was impaired. These controversial results were later partially explained by a demonstration that neutrophils adhering to plastic dishes underwent significant increases in oxidative metabolic activity following endotoxin challenge, whereas those maintained in suspension failed to do so (Dahinden and Fehr, 1983). Surface contact therefore may be an important determinant of neutrophil responsiveness to certain stimuli including LPS.

Effect on neutrophil production: Bacterial LPS induce dramatic haematologic and haemodynamic changes following intravenous administration of these toxic components into animal species including man (Territo and Golde, 1976). These changes are reminiscent of those clinical symptoms observed in gram-negative septic patients. One of the earliest observable haematologic aberrations after LPS injection is a transient disappearance of circulating neutrophils or neutropenia. Neutrophilia or an increase in neutrophil production ensues immediately following the neutropenic state as a result of a proliferative response and release of mature cells from the bone marrow due to endotoxin challenge.

Section 8

Diabetes mellitus

In the untreated state diabetes mellitus is recognized by chronic elevation of glucose concentration in blood (hyperglycemia). It is a heterologous primary disorder of carbohydrate metabolism with multiple etiologic factors that generally involve absolute or relative insulin deficiency or insulin resistance or both. The severity of its symptoms is largely determined by the degree to which insulin action is deficient. The currently accepted classification and criteria for diagnosis of diabetes are based on the 1979 report of the National Diabetes Data Group (NDDG, 1979).

Diabetes can be separated into 2 general disease syndromes:

(1) type I or insulin-dependent diabetes mellitus (IDDM) is present in patients with little or no endogenous insulin secretory capability. With extreme hyperglycemia developing, they are entirely dependent on exogenous insulin therapy for immediate survival. This form of diabetes usually but not always develops in early childhood. Older terms for this syndrome are ketosis prone, brittle diabetes or juvenile onset.

(2) type II or non-insulin-dependent diabetes mellitus (NIDDM) manifests in patients who retain significant

endogenous insulin secretory capacity. Although treatment with insulin may be necessary to control hyperglycemia, patients do not develop ketosis in absence of insulin therapy and are not very dependent on exogenous insulin for survival. Previous terms for NIDDM include maturity or adult onset, nonketotic and stable diabetes.

In addition to the major categories of diabetes, large number of secondary forms have been identified. They exist when some other primary pathophysiologic states are strongly associated with the diabetic state. In general these cases are unusual and comprise only a small proportion of the total diabetic incidents. Any disease process that limits insulin secretion or impairs insulin action can cause secondary diabetes.

Pathogenesis

A strong genetic component is said to be involved in the etiology of IDDM. A major association with HLA antigen particularly DR3, DR4 as well as HLA DQ has been suggested (Wolf et al, 1983; Henson et al, 1986; Michelson and Lernmark, 1987). There are also several lines of evidence suggesting a role for viruses (Yoon et al, 1979; Rubenstein et al, 1982). Circulating autoimmune antibodies binding to cytoplasmic antigens of islet cells and B cell surfaces are detectable in patients blood implicating their part in initiating the

disease state (McCuish et al, 1974; Betterle et al, 1987).

Abnormalities of insulin and to a lesser extent glucagon secretions and actions are central to the pathogenesis of NIDDM (Lang et al, 1981; Leslie et al, 1986). Insulin deficiency, resistance, and accelerated hepatic glucose production existing in NIDDM are considered to be contributing to hyperglycemia in the patients (Donner et al, 1985; Reaven et al, 1985; Swislocki et al, 1987).

Host defense mechanisms in diabetes mellitus

Patients with diabetes are considered to be more susceptible to infections which seem to be more severe and difficult to control than in non-diabetics (Rayfield et al, 1982). Various steps involved in host response to invading microbes such as PMN chemotaxis, adherence to endothelium, phagocytosis, intracellular killing, and cell-mediated immunity have been found to be depressed in some diabetic patients (Mowat and Baum, 1971; Bagdade et al, 1974; Tan et al, 1975, Tater et al, 1987; Andersen et al, 1988). It is still unclear if these defects actually contribute to enhanced susceptibility to infections in diabetic subjects. A combination of defects in a patient with poorly controlled diabetes may well be synergistic and together those defects can

either heighten susceptibility or augment severity once infection is initiated.

Neutrophil function

One of the earliest studies to examine the nature of lowered bacterial resistance in diabetics was conducted by Marble and co-workers (Marble et al, 1938). Using whole blood from 27 diabetic patients and 23 normal control subjects, they documented similar phagocytic, bacteriostatic and bactericidal power against selected strains of streptococci among these samples. They explained the negative results on the basis of well controlled disease state without presenting any data to evaluate the degree of diabetic control and studies of other organisms that might be of clinical relevance to the patients. To understand the role of neutrophils in diabetics, a study of metabolic aspects of the cell was undertaken (Esmann, 1983). PMNLs from severely diabetic patients exhibited a decreased rate of glycolysis due to decreased activity of phosphofructokinase. A decrease in total activity of glycogen synthetase and impaired activation of this enzyme led to a decrease in glycogen synthesis thereby reducing the glycogen content of the cell. It is hypothesized that such enzymatic impairment is crucial in diabetic patients and can be restored upon insulin treatment.

Chemotaxis

Perillie and colleagues studied the local exudative response to inflammation in the skin of diabetic patients using the Re buck glass coverslip technique (Perillie et al, 1962). During the first 3 hours the neutrophil response was virtually absent in patients with acidosis (uremic or diabetic) in comparison with non-diabetic and well controlled diabetic subjects. Local cellular responses were markedly increased with improvement of acidosis thus concluding that acidosis resulted in reduced capacity to resist invasion and spread of bacterial organisms. Kjersem et al (1988) made a direct study of the correlation between blood sugar level and neutrophil motility. PMN migration measured both in vivo (skin-chamber) and in vitro (modified Boyden chamber) was shown to be decreased in diabetics during normo- as well as hyperglycemic states. Short-term changes in metabolic control seem to be without effect on chemotactic abnormality. Poor PMN movement has been described not only in diabetic patients but also in their non-diabetic first degree relatives suggestive of a heritable defect rather than one induced by metabolic upset (Molenaar et al, 1976).

Phagocytosis

In vitro phagocytosis examined in 38 nonacidotic and 7 acidotic diabetic patients showed that only the

latter manifested decreased phagocytic activity and the defect disappeared upon correction of acidosis (Bybee and Rogers, 1964). Recent experimental evidence however showed that phagocytosis in diabetic patients was comparable to that of control cells unless the control cells were incubated with the patients sera which tended to reduce the activity (Tater et al, 1987). ~~Later~~ Kjersem et al in 1988 observed that serum independent phagocytosis was significantly increased during normo- and hyperglycemia but a reduction was noted from normo- to hyperglycemia. Serum dependent phagocytosis of opsonized LPS particles however was no different in patients and controls during normoglycemia.

Intracellular killing

Nolan et al (1978) separately assayed engulfment and intracellular killing of *S. aureus* in an in vitro system using diabetic PMN. The cells treated with insulin or oral hypoglycemic agents displayed decreased bacterial killing as compared to the controls. When the patients were retested after improved blood glucose-control, 6 out of 7 patients showed improvement in intracellular killing. Later oxidative killing of *Candida albicans* in vitro under elevated glucose and beta-hydroxybutyrate concentrations both independently and in combination was studied (Wilson and Reeves, 1986). Their data suggest that oxidative killing by

neutrophils from IDDM patients is inhibited by high concentrations of glucose and ketones be it separately or in combination. Killing by non-oxidative means was nevertheless unaffected by the in vitro metabolic disturbances.

Adhesion

Diabetic neutrophils manifested diminished adherence to nylon fiber columns (Bagdade et al, 1978) and later were shown to have a similar physiological impairment when bovine aortic endothelium was employed as a substratum for adhesion (Anderson et al, 1988). No correlation between metabolic control and adherence to the endothelium was suspected but instead the impaired release of fibronectin by neutrophils was suggested to be the explanation for defective adhesion. They found that diabetic plasma augments adherence of diabetic neutrophils to endothelium implicating a plasma factor as acting specifically on endothelium rendering the substrate hyperadhesive.

Accumulated data allow a formulation of a model which may account for the infectious complications of diabetes. Diabetic PMNs have defective adherence to endothelium resulting in a poor tissue inflammatory response although in the intravascular space the plasma may serve to correct the defective property. There is a speculation that 2 factors, defective neutrophil adhesion and

endothelial cell hypoadhesiveness, together may act to prevent PMN exudation from the vascular space and hence results in a suboptimal neutrophil response in inflammation.

Section 9

Purpose of study

The ability of PMNs to migrate towards a site of inflammation is an area in cell biology that has long been of interest to cellular immunologists. The work in this thesis is intended to contribute further to the knowledge of the mechanism by which these cells traverse the blood vessel wall towards the site of injury particularly in the presence of endotoxins. The project to be described will mainly focus on the locomotory responses of PMN when challenged with LPS and the effects of certain antagonists on these responses. Antagonists used included polymyxin B, steroidal and non-steroidal anti-inflammatory drugs. Monoclonal antibodies towards CD18 glycoproteins were used to observe the blocking effect on neutrophil shape change due to LPS and eventually to establish whether this family of receptors plays a role in providing a receptor site for LPS on the cell surface which controls the locomotor response to LPS. To assay the locomotory responses of PMN, recent techniques in studying cell locomotion were implemented namely the polarisation assay, the micropore filter assay, the visual assay and the gel invasion assay.

Once data had been obtained from normal healthy individuals, the system was tested on cells from

patients. PMN from diabetic subjects were chosen for this study since PMN functions in these patients have been reported to be defective (see previous section). Besides investigating the locomotive function, metabolic activity of these cells in comparison with the normal cells was evaluated in the presence of LPS using a chemiluminescence assay.

CHAPTER 2

MATERIALS AND METHODS

Siliconization of glass slides

Siliconized cavity glass slides were used to study the PMNL activity in suspension observed on a time-lapse video system. The slides were initially cleaned by dipping them in stericol solution and washed with running water. They were then air dried and cooled before siliconizing them. To coat with silicone, the slides were immersed in repelcote (Hopkin and Williams, Chadwell Heath, England) a 2 % solution of water repellent consisting of dimethyl-dichlorosilane in 1,1,1-trichloroethane, for 1 minute and then air dried for another 60 minutes. This was done at room temperature inside a fume hood. After drying, the slides were rinsed with distilled water to remove any traces of hydrochloric acid formed during hydrolysis of repelcote. Finally the slides were dried in a hot air oven and then autoclaved for sterilization.

Preparation of standard buffer

For a standard buffer, one molar MOPS (3-(N-morpholino) propanesulfonic acid) (Sigma, Poole, Dorset) was prepared by dissolving 20.93 gm in 90 ml of distilled water with a magnetic stirrer. The pH of the buffer was adjusted to 7.4 using the pH meter by slow addition of 16N sodium hydroxide (BDH Chemicals Ltd, Poole, England) with constant stirring. The final volume

was adjusted to 100 ml at the desired pH value followed by filter sterilization. The latter was done by filtering through 0.2 μ m pore sized filters (Flow- Pore D26, Flow Labs Ltd., Rickmansworth, Herts, England), and the solutions were aliquoted into 20 ml sterile universal bottles and stored at 4° C before used.

Preparation of media

Hanks balanced salt solution (HBSS) (Flow Labs Ltd., Rickmansworth, Herts) was prepared by diluting a ten-times strength HBSS solution (10 ml volume) with 89 ml of sterile distilled water and 1 ml of 1M MOPS. This medium was labelled as HBSS/MOPS after adjusting the pH to 7.2 with 1N NaOH. HBSS phenol red free solution was used as a prepared isotonic solution (Flow Labs) and buffered by the addition of 1 ml 1M MOPS to 99 ml HBSS. Human serum albumin (HSA) (Hoechst, England) was dissolved in HBSS/MOPS or phenol red free HBSS/MOPS (prf-HBSS/MOPS) at a concentration of 10 mg/ml.

Preparation of fixative

Glutaraldehyde (Grade II, 25 % aqueous solution, Sigma, Poole, Dorset) was added to HBSS/MOPS to give a 2.5 % v/v solution. The pH was adjusted to 7.4 by adding 1N NaOH. The fixative used to fix cells throughout the project where appropriate was designated as glutd/HBSS-MOPS.

Preparation of test reagents

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), leukotriene B₄ (LTB₄), luminol, phorbol 12-myristate 13-acetate (PMA), zymosan and dimethyl sulphoxide (DMSO grade I) were purchased from Sigma. FMLP, LTB₄, luminol and PMA were prepared as stock solutions of 10^{-2} M in DMSO and stored at -20° C. Fresh FMLP solution at desired concentrations were made up in HBSS/MOPS when needed. LTB₄ was used in thin layer chromatography as prepared. Luminol and PMA were diluted at appropriate concentrations in prf-HBSS/MOPS before used in chemiluminescent assay.

Non-steroidal antiinflammatory drugs BW755C (Burroughs-Wellcome) and Revlon 5901 (ICI), were given by Dr. A. Rossi (Pharmacology Dept., Glasgow University) while indomethacin and dexamethasone were obtained from Sigma. The drugs were dissolved in DMSO at a concentration of 10^{-2} M.

Peptides possessing the triplet RGD sequence were purchased from Sigma: Gly-Arg-Gly-Asp-Ser, Gly-Arg-Gly-Asp-Pro-Lys, Arg-Gly-Asp and Arg-Gly-Asp-Ser. These peptides were dissolved in HBSS/MOPS at a concentration of 10^{-2} M. Monoclonal antibodies against CD11/CD18 glycoproteins, adhesion related surface receptors of

leukocytes, were generously given by Prof. A. McMichael and Dr. D. Mason (University of Oxford) and Dr. N. Hogg (Imperial Cancer Research Fund, London). The antibodies (listed in table 1) were dialysed against HBSS/MOPS for 24 hours to remove azide and eventually stored in a -20° C freezer at $1\text{ }\mu\text{g}/\mu\text{l}$ concentration.

Platelet activating factor, PAF C_{16} (1-O-Hexadecyl-2-acetyl-sn-glycero-3-phosphocholine), was obtained from BCL, Sussex, England. The factor was dissolved in HBSS/MOPS at 10^{-2}M . Glucose, anhydrous monosaccharide sugar, was supplied by BDH Chemicals, Dorset, England.

Lipopolysaccharides of *Shigella flexneri* (Ra, Rc, Sla and Slc) were prepared by Dr. Simmons (Dept. Bacteriology and Immunology, Western Infirmary) while lipopolysaccharides of *Salmonella minnesota* (Re and Sm) were purchased from Sigma. These lipopolysaccharides (LPS) were from rough strain mutants (designation 'R') and smooth strains (designated as 'S') of the bacteria. Figure 2 shows the exact positions of these LPS chemotypes. The LPS were mixed in a 56° C sterile distilled water at a final concentration of 1 mg/ml and kept at 4° C. Lipid A from *Salmonella minnesota* was purchased from Sigma and prepared similarly to LPS. Freeze-dried polymyxin B sulphate (Aerosporin, Wellcome

Anti-CD11a	Anti-CD11b	Anti-CD11c	Anti-CD18	Donor
711	649	256	706	McMichael
714	650	277	710	"
715	651	300	713	"
717	652	721	719	"
720	653	727	725	"
723	654	728		"
724	656			"
	657			"
	44	3.9		Hogg
	2LPM19C	KB23		Mason
		KB90		"

Table 1. Antibody codes. Monoclonal antibodies used to block LPS-induced PMN polarization.

Foundation Ltd., England) was dissolved in HBSS/MOPS at a concentration of 1 mg/ml.

Fresh human serum

Blood samples from normal and patient volunteers were transferred into sterile bijou containers and left undisturbed for 30 minutes at room temperature for clotting. The clot was centrifuged at 500 X g for 5 minutes and the clear serum was saved in a plastic tube.

Isolation of neutrophil leukocytes

Human blood was collected from normal donors by venepuncture and was mixed with preservative-free heparin (Evans Medical Ltd., Liverpool) at 10 units/ml in sterile plastic universals (Sterilin Ltd., Teddington, England). Patient's blood samples were generally transported in a sterile lithium heparinised plastic tube. The patients involved in the study were of type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetics. Their ages ranged from 20 to 40 years and the duration of the diabetes was between 1 and 28 years. All of the subjects were microproteinuria negative and had no infections during the study while approximately half had background diabetic retinopathy. The blood was then layered onto lymphocyte separation medium, an aqueous solution of Ficoll and sodium metrizoate with a density of 1.077 gm/ml at 20° C (Flow

Labs Ltd., Rickmansworth, Herts), in a ratio of 2 ml of separation mixture to 3 ml of blood. The content was then centrifuged for 30 minutes at 400 X g. All centrifugations were carried out at room temperature and sterile conical base plastic centrifuge tubes were used throughout the experiments (Sterilin Ltd., Teddington, England). The supernatant including the mononuclear cell layer was removed and the mixture of erythrocytes and granulocytes was resuspended in HBSS/MOPS. This suspension was mixed with 6% dextran solution (Dextraven 110, CP Pharmaceuticals Ltd., Wrexham, UK) in a proportion of 1 ml dextran to 3 ml of cell suspension, left for 40 minutes at room temperature and the leukocyte-rich supernatant was then decanted. Erythrocyte contamination is conventionally removed by the use of aggregating agents such as high molecular weight dextran (Skoog and Beck, 1956). These agents promote aggregation of erythrocytes in fluid medium consequently leading to more rapid sedimentation rate of the red blood cells. The leukocyte-rich supernatant was centrifuged for 10 minutes at 400 X g leaving the pellet comprising of granulocytes and some erythrocytes. Removal of residual erythrocytes contamination is of great importance in this project especially in chemiluminescence studies as red coloured solutions may quench light emission. Erythro-lysis of the leukocyte-rich pellet was performed by osmotic lysis in which the

cells were briefly exposed to distilled water. The procedure effectively removes erythrocytes but must be done rapidly (< 30 seconds) to leave the leukocytes intact. The suspension was transferred to another tube followed by adjustment of the solution to isotonicity with the addition of HBSS/MOPS and centrifuged at the above mentioned speed and time. The pellet was washed one more time before resuspending in HBSS/MOPS containing HSA and counted in an improved Neubauer haemocytometer. The yield ranged from $1.0-2.0 \times 10^7$ cells/ 10 ml of blood giving a population of $> 95\%$ round neutrophils as judged by phase-contrast microscopy with a 40X objective.

Checking cell viability

A 0.2% solution of trypan blue (BDH Chemicals Ltd., Poole, UK) was prepared in normal saline and was filtered to remove any undissolved dye. One hundred microliter of cell suspension at 10^6 cells/ml was mixed well with 100 μ l of the dye solution and left for 1 minute at room temperature. A drop of this mixture was placed on a microscope slide, mounted with a coverslip, examined under the 20X phase-objective of an inverted microscope (Nikon, MSD). Viable cells excluded the dye solution and were refractile whereas dead cells took up the dye and stained blue. Neutrophils isolated by the stated procedure always contained $> 97\%$ viable cells.

Isolation of collagen

Type 1 collagen was prepared from rat tail tendons following the method of Elsdale and Bard (1972) with slight modifications . Tails were collected from rats weighing 230-250 grams. They were soaked in 70% ethanol for 5 minutes before removing the skin. Tendons were stripped and dipped in HBSS/MOPS containing 50 units/ml penicillin and 50 μ g/ml streptomycin (Flow Labs Ltd., Rickmansworth, UK) for 5 minutes. The medium was removed and the tendons were soaked in 3% acetic acid (BDH Chemicals Ltd., Poole, UK) in a sterile conical flask and left overnight at 4° C. The content of the flask was thoroughly mixed for 1 hour with a magnetic stirrer and undissolved materials were removed by centrifugation at 800 X g for 30 minutes. The clear tropocollagen solution was mixed with 20% wt/v sodium chloride at 1:2 to precipitate the collagen. It was then centrifuged at 600 X g for 40 minutes when collagen precipitates accumulated at the top of the centrifuge tube and the undissolved materials were removed as a pellet. Collagen precipitates were collected and washed once with distilled water. They were dissolved again in 3% acetic acid and centrifuged twice at 800 X g for 20 minutes each time rejecting a portion of the solution at the bottom of the centrifuge tube. This treatment completely removed small undissolved materials from the collagen

solution with an increase of optical quality. Collagen solution was dialysed against distilled water adjusted to pH 4 with hydrochloric acid. The concentration of collagen in solution was determined by comparing the optical density value at 234 nm (using matched cuvettes in a Pye Unicam Spectrophotometer, SP 1800) to a standard graph prepared with a freeze dried sample of the same collagen preparation. Aliquots of the collagen solution were dispensed into sterile plastic tubes and stored at -20° C until use.

Polarisation assay

The assay depends on obtaining a starting population of round unstimulated cells. Conical bottomed plastic tubes were used throughout as prolonged contact with glass causes adhesion and eventually release of endogenous chemotactic factors leading to a high background of polarised cells. After the process of cell separation, neutrophils were checked for their polarised morphology. Final cell preparations should be > 95% spherical. Otherwise the preparation had to be discarded.

For the assays, following the method of Haston and Shields (1985), neutrophils were adjusted to 1×10^6 cells/ml in HBSS/MOPS+HSA to which the substance to be tested was added. The tubes were incubated for the required time period at 37° C and then equal volumes of

2.5% glutd/HBSS-MOPS were added to each tube to fix the cells. After 10 minutes the fixed cells were washed twice in HBSS/MOPS and resuspended in the remaining medium (about 500 μ l). The fixed cells were examined by phase contrast microscopy with a X 40 objective (Vickers Microscope) and any cells deviating from spherical outline were scored as polarised. A total of 300 cells were counted in each preparation and the number of polarised cells was expressed as a percentage of the total cells enumerated. Neutrophils which were spherical in outline but with filamentous projections or small blebs were counted as negative or unpolarised.

Supernatant collection

Neutrophils at 1×10^6 /ml were incubated in sterile conical test tubes containing 10 μ g/ml LPS for 90 minutes at 37° C. The tubes were then centrifuged at 400 X g for 8 minutes and the supernatant was transferred to another tube before being used. With this simple method, whatever factor(s) that are being released by the cells should be obtainable in the cell-free supernatant. The supernatant was usually used immediately in appropriate tests with a new batch of neutrophils or sometimes stored at 4° C overnight before use.

Preliminary investigation of released factor(s)

The supernatant obtained from preincubating neutrophils with LPS was dialysed against HBSS/MOPS for 24 hours at 4°C. The dialysate and the solution outside the dialysing tubing were tested for the presence of factor(s) that stimulate locomotion as judged by the polarisation assay. The supernatant was also subjected to heat treatment at 56° C for 30 minutes or 100° C for 3 minutes.

Hexane extraction and thin layer chromatography

An equal volume of supernatant and hexane was mixed in a separating funnel for the liquid-liquid extraction process. The aqueous layer was eluted and mixed with another volume of hexane twice after which it was restored to physiological conditions and tested for the presence of any neutrophil polarising activity. The organic layer was collected, pooled together and concentrated using a rotary evaporator (Büchi 1.011, Switzerland). Evaporation was conducted under pressure provided by a water pump. The evaporation flask was heated to a temperature of 50° C on a water bath and rotated during the process.

The concentrated organic layer was spotted on a thin layer chromatographic plate which had been coated with silica. The plate was then placed in a chamber containing 20% ethyl acetate and 80% petroleum ether for

60 minutes at room temperature. Later the plate was air-dried, sprayed with concentrated hydrochloric acid and heated in a 250° C oven for 5 minutes. Before spraying, the plate was usually observed under ultraviolet light to spot the probable area where the substance had migrated. The concentrated sample was also tested for neutrophil polarising activity under physiological conditions.

Gel filtration and spectrophotometry

A column of Sephadex G-25 (superfine) (Pharmacia Fine Chemicals, Middlesex, UK) was prepared by allowing the dry powder at 5 mg/ml to swell in an excess of phenol red-free HBSS/MOPS for 1 hour at 90° C. The high temperature serves to accelerate the swelling process as well as to deaerate the buffer. The suspension was stirred gently with a glass rod. Once the gel had swollen, it was carefully poured into a 25 ml (1 cm in diameter) chromatography column. The column, with a stopcock plug at the bottom, was plugged with cotton wool to form a bed support for the gel suspension. All the gel required was poured in a single operation and was allowed to settle before opening the outlet. The outlet plug was released to allow the eluant to flow out with three volumes of eluent passing through the column in order to stabilize the bed. Each time the buffer was eluted out, the top portion of the column was carefully

filled with eluant buffer thus preventing the column from drying and disturbing the packing process. After the third running, the sample was layered above the gel surface using a pasteur pipette and the column outlet was opened allowing the sample to drain into the bed. The eluant was collected in sterile plastic tubes at a 1 ml volume each. The samples collected were then analysed for protein content by spectrophotometric determination before using it in polarisation assay. Matched cuvettes in a Pye Unicam Spectrophotometer SP 1800 were used to determine the absorbance of protein molecules at 280 nm wavelength.

Micropore filter assay

As mentioned in the introduction, the assay consists of an upper chamber suspended in a lower chamber. The upper chamber is a sawn-off tuberculin syringe barrel while the lower chamber is a machined multiwell plastic compartment placed in a box with holes bored in its lid. Cellulose ester filters (Millipore, Bedford, UK) with a thickness of 150 μm and pore size 3 μm were glued to the lower end of the upper chamber with Uhu glue (Beecham Uhu, Middlesex, UK). The lower compartments were filled with 170 μl of test reagents and the upper chambers contained 1×10^6 neutrophils/ml in HBSS/MOPS+HSA in 100 μl . The filters were allowed to wet from the top before lowering into the lower chambers

to ensure that the filters were presoaked with non-chemotactic medium. The tests were incubated at 37° C for 60 minutes undisturbed to allow cell locomotion.

At the end of the incubation period, the upper chambers were removed, inverted to empty out the fluid and were immersed in 70% ethanol to fix the cells present on and in the filters. As the glue melted the filters were detached from the barrel and transferred to Sterilin plastic dishes containing 25 compartments each of 1.8 cm² using a forceps touching only the rim. Small holes were punched out at the bottom of each compartment allowing fluid to flow in and out freely. The filters were well identified by placing them in different compartments before commencing the staining step. The filters were stained in sequence by dipping them in distilled water for 1 minute, haematoxylin 1 minute, distilled water 1 minute, tap water 10 minutes, 70% ethanol 2 minutes, 95% ethanol 3 minutes and a mixture of 80% ethanol and 20% butanol for 5 minutes. Since xylene dissolves plastics, dipping the filters in xylene was carried out in glass bijou bottles. The filters were mounted top side up on glass slides covered with DPX and coverslips after the xylene had clarified the filters. To assess cell migration, the leading front method was used. Using the X 40 objective of a light microscope, the micrometer fine adjustment was racked down past the leading front of cells. It was then racked back up to

the leading plane in which the nuclei of two or more cells were in focus together within a field, at which a micrometer reading was taken. The fine adjustment was racked up to the top of the filter and a second micrometer reading was noted. The difference between the two readings gave the measure of the migrated distance by the leading front of the cells. A mean was calculated after five readings were taken at different planes in each filter.

Preparation of collagen gel assay

Three-dimensional collagen matrices were prepared by adjusting the osmolarity and pH of the stock collagen solution, 2 mg/ml, to physiological levels (Shields et al, 1984). One milliliter of 10X RPMI 1640 (Flow Labs Ltd., Herts, UK), 200 μ l MOPS (pH 7.4), 44 μ l NaOH and 8.8 ml of collagen solution were mixed rapidly in a sterile plastic tube (final collagen concentration in the gel was 1.76 mg/ml). The method to study human neutrophil migration towards gradient sources was adapted from Islam et al (1985). Gels for the chemokinetic experiments were prepared by dispensing 0.5 ml portion of the mixture into the wells of a tissue culture plate (Flow Labs) with 24 flat-bottomed wells (1.7 X 1.6 cm). Gels were allowed to become firm at room temperature within 15 minutes. The substance to be tested was layered onto the gel and left for 1 hour for

even distribution. The remaining fluid was removed and cells in suspension containing the tested substance were overlaid on top of the gel. For controls, the substance was absent in both the gel and cell suspension. Neutrophils suspended were used at 10^6 cells/well in 1 ml suspension.

Gels for the chemotactic assays were prepared as follows: 0.4 ml of collagen was distributed and allowed to set in wells of tissue culture plate. The test substance was layered on top of the gel for 1 hour after which the remaining fluid was removed followed by layering of 0.3 ml collagen over the first gel and allowed to set. This was to form a layer through which the substance could diffuse to form a gradient. A suspension of neutrophils at 10^6 cells/ml in HBSS/MOPS + HSA was added to the top of the collagen layer an hour later. A similar procedure was used for controls with the absence of test substances. The plates were immediately incubated in moist chambers at 37° C for the cells to invade the gel for various time periods. Collagen gels were fixed after the prescribed time with 2.5% glutd/HBSS-MOPS for 30 minutes at room temperature. The upper surface of the gel was washed 3X with HBSS/MOPS to remove non-invaded cells. A leading front method was used to quantify the infiltration of neutrophils into the gels (Zigmond and Hirsch, 1973). Four randomly chosen fields on duplicate gels were counted for

migrated neutrophils assessed by the calibrated micrometer of a Nikon inverted phase contrast microscope under X 200 magnification.

Visual assay of neutrophil shape change in suspension

The filming chamber used for visual assay of cells in suspension was a siliconised cavity glass slide (76 X 25 mm; BDH Ltd., Glasgow). Square coverslips (22 X 22 mm) were washed in sterical, rinsed thoroughly in distilled water and finally blot dried with soft tissue. A sample of 100 μ l of cells at 10^6 cells/ml in HBSS/MOPS+HSA preincubated at 37° C with 10 μ g/ml LPS was transferred to the chamber after each incubating time period covered with a clean coverslip that had silicone vacuum grease ringed around it. The filming chamber was immediately placed on an Olympus phase-contrast microscope stage (BH-2) at a X40 magnification where the temperature was maintained at 37°C using a warm air curtain. After filming was done using a Panasonic video camera and a time lapse recorder (Panasonic NV-8050) recording at 48 times faster than real time, shape change was traced out from the screen on to a tracing paper by playing back the recorder and pausing for every 30 seconds to draw the outlines.

Shape analysis of fixed cells

Glutaraldehyde-fixed cells after polarisation assay were mounted on slides and the outlines of the cells were drawn using a camera lucida attachment on a Wild M20 microscope with a bright field illumination and a X100 objective. The optical section that most nearly represented the shape that could be deduced by focusing up and down was drawn. With the assistance of Dr. J.M. Lackie from the Department of Cell Biology, Glasgow University, outlines of the cells were then traced on a digitizing tablet with the stylus set in a stream mode, a technique introduced to analyse cell behaviour(Wilkinson et al, 1988). The three shape parameters (elongation, dispersion, and extension) were stored for each cell and at least 50 cells were measured from each experiment. This analytical exercise was first used by Dunn and Brown (1986). Extension is a measure of how much the shape differs from a circle and has a value of zero if the shape is circular. It is also the sum of elongation and dispersion. Dispersion is a measure of irregularity of contour and proved useful as an objective criterion to distinguish spherical from non-spherical cells. Elongation is a measure of how much the shape must be compressed along its axis in order to minimize its extension. Elongation can never take a value of less than zero or greater than extension. Comparisons between values were made using Mann-Whitney U-tests.

Chemiluminescence

Cells for luminometry were prepared as described previously but were finally resuspended in phenol red-free (prf)- HBSS/MOPS +HSA. Opsonization was performed by suspending zymosan in prf-HBSS/MOPS containing serum at appropriate dilution and incubating at 37° C for 30 minutes in a water bath. The suspension was centrifuged at 3000 X g for 10 minutes before washing the pellet twice and finally resuspended in prf-HBSS/MOPS. Luminol, used to amplify chemiluminescence, which had been dissolved in DMSO at 10^{-2} M as a stock solution was further diluted in prf-HBSS/MOPS to the required concentration prior to use.

The cell suspension, luminol in medium and test substances were pre-warmed at 37° C in a waterbath for 10 minutes before using them. The reaction mixture consisted of neutrophil suspension (10^6 cells/ml), 200 μ l luminol at 10^{-4} M and test substances mixed in a 3 ml disposable test tubes (LP3, Luckham Ltd., Sussex). It was placed in the light-proof chamber of the Luminometer 1250 (LKB Wallac). The carousel was rotated to bring the sample in line with the photomultiplier tube to record the readings; the resulting light output in mV was recorded on the digital printout set at 10 second recording integrals. The temperature within the reaction tube was controlled by water passing from

thermostatically controlled circulator through a polished hollow metal sample holder thus maintaining the reaction mixture at 37° C. The gain control was set to give a reading of 10 mV using a built-in standard while a background subtraction control zeroed the instrument prior to placing the sample in the chamber.

Photomicrography

Still photographs were taken using an Olympus microscope (BH-2) equipped with phase-contrast optics with X40 and X100 objectives. Photographs were taken on Kodak Panatomic X 35 mm films (ASA 32). The films were developed using HC110 developing solution and fixed with Unifix. For printing on Kodagraph projection paper, Kodak Dektol developer and Unifix were used.

Statistical analysis

Each experimental value was determined in triplicates or more or otherwise stated. The average values were used for statistics. Data were expressed as means \pm standard deviation values. Differences between the controls and test samples were analysed by the two-tailed Student's t-test for paired data (significant $P < 0.05$). Where appropriate, correlation coefficients were determined and the significance test was made using the t-test. Non-parametric tests. Mann-Whitney U-tests, were used in the shape analysis of fixed cells.

CHAPTER 3
RESULTS AND DISCUSSION

Section 1

POLARISATION ASSAY

Besides the widely known chemotactic factors, the presence of LPS causes polarization in a suspension of PMN (Haslett, 1985). In the first part of the project, LPS from *Shigella flexneri* and *Salmonella minnesota* were tested in the polarisation assay using several different LPS chemotypes to investigate the relationships that might exist between the structural complexity of LPS and the PMN shape change in the absence of serum. Figure 1 shows the cell envelope of Gram-negative bacteria and a detailed structure of the envelope. The general structure of bacterial LPS with the different sites of mutation resulting in the chemotypic variations is shown in figure 2. Gram-negative bacteria appear to share a common pattern in the structure of their cell envelopes (Hammond et al, 1984).

Dose response

After isolating PMN from the whole blood, the cells were incubated with varying concentrations of LPS of each chemotype at 37° C for 60 minutes and then fixed with 2% glutaraldehyde. The number of cells acquiring polarised morphology was enumerated and expressed as a percentage. The rough strain LPS (Ra and Rc of *Shigella*

flexneri, and Re of *Salmonella minnesota*) of the bacterial species caused a greater percentage of shape change in neutrophils (Figure 3) than the smooth or wild type strains (Sm of *Salmonella minnesota*, S1a and S3a of *Shigella flexneri*). The percent polarisation increased as the concentration of LPS increased and eventually plateaued out with an optimum at 10^4 ng/ml. Beyond this concentration there was no enhancement of polarisation. At the lower level of LPS concentrations, both the rough and smooth strains were able to elicit shape change down to 100 ng/ml. Within the rough strain chemotypes, the Re mutant of *Salmonella minnesota* induced more polarization (54%) than the two chemotypes of *Shigella flexneri* (30% - Ra, 37% - Rc) while among the smooth strains there was less variation (18-25% polarisation).

Time course

Using 10^4 ng/ml ($10 \mu\text{g/ml}$) as the working concentration, LPS from *Salmonella minnesota* Re mutant was assayed for its effect on shape change at times between 0 to 110 minutes. Figure 4 shows that the proportion of polarized cells increased to a maximum at 90 minutes. Prolonging the time of incubation up to 150 minutes failed to increase the number of PMN polarising in response to the presence of LPS. Figure 5 shows a series of pictures of neutrophils at different times

after exposure to LPS. Some cells showed polarization after 15 minutes of exposure to LPS (Figure 5c) and this increased up to 60 minutes (Figure 5d-f). Cell viability remained intact in LPS up to 110 minutes judged from the the morphology and refractility of the cells under phase-contrast microscopy.

Effect of antibiotic

Polymyxin B sulfate has been frequently used to counteract the biological effects of LPS by forming a stable complex with the lipid A region of LPS molecule (Morrison and Jacobs, 1976). Hence, in this polarising assay polymyxin was added to neutralise any LPS effects on the neutrophils. Varying concentrations of polymyxin were mixed with the cells for 10 minutes before challenging with LPS for 90 minutes fixing the cells at the selected incubating period. Equivalent concentration of polymyxin to that of LPS (10 $\mu\text{g/ml}$) did reduce the population of cells with non-spherical shape but statistically this was insignificant (Figure 6). Smaller concentrations were virtually ineffective in modulating the polarisation of PMN. Incubating LPS with polymyxin before adding the cells did not produce a similar result to that shown in figure 6; cells polarising as if there was no antibiotic present. Incubation of cells with polymyxin alone did not cause any shape change thus

excluding the possibility of the antibiotic contributing to the polarisation of PMN.

Discussion

The effect of LPS on neutrophil shape change has been reported (Haslett et al, 1985) but not examined in detail. Such neutrophil responses were therefore investigated employing different LPS chemotypes of bacterial species. Figure 3 suggest that LPS structure is important in affecting shape change of the neutrophils. As the number of sugar moieties decreased, a bigger polarising response was observed supporting the view that the molecular composition of LPS partially determines the polarisation effect. This relationship can be seen from figure 7 which depicts the polarising activity of PMN with respect to the specified chemotypic variation of LPS molecule. Thus these sugar moieties may prevent LPS from interacting with the neutrophils. This observation is similar to that of the phagocytic activity of PMNs towards a bacterium in which smooth strains of a bacterial species having complete polysaccharide chains are less effectively phagocytosed than mutants devoid of certain sugar molecules on the cell wall backbone (Hammond et al, 1984). It has been documented that the mutant Re 595 endotoxin, which is devoid of all O- and R-polysaccharides and backbone sugar heptose, does possess similar biological

activities including immunogenicity, pyrogenicity, and lethality to that of the smooth LPS though somewhat lower (Kim and Watson, 1967; Kasai and Nowotny, 1967). It is quite evident then that O-polysaccharides as well as R-polysaccharides and heptose are either non-functional in inducing the shape change of neutrophils or inhibit shape change suggesting that the shape-change response is associated with the lipid portion of the molecule.

Unlike the shape change in response to chemotactic peptides that occurs rapidly (between 5 to 30 minutes), polarisation of PMN after the exposure to LPS was slow. Maximal response was attained only after 90 minutes of exposure. The slow response suggests an indirect effect of LPS which may prime the neutrophils to respond to some other factors. To define the requirement of the presence of LPS in inducing shape change, the neutrophils were pretreated with polymyxin B sulfate, an antibiotic which has been reported to diminish the biologic effects of endotoxin on neutrophil lysosomal enzyme release, metabolic changes associated with the release and reduction of nitroblue tetrazolium dye (Bannatyne et al, 1977; Corrigan et al, 1974). The neutralising effect of polymyxin on LPS-induced neutrophil shape change was tested. The number of polarised PMN was minimally reduced (figure 6) and this was insignificant statistically. Polymyxin B sulfate has been shown to disrupt bacterial endotoxin (Koike et al,

1969). Earlier studies proposed that polymyxin competes for the same receptor sites as that of LPS on the target cell (Kunin, 1970; Kunin and Bugg, 1971). Later Morrison and Jacobs (1976) reported that polymyxin in fact interacted directly with the lipid A region of bacterial LPS. They have demonstrated the interaction to be stoichiometric. The ineffectiveness of polymyxin in causing significant blocking of cell polarisation could be due to the presence of a second (non-LPS) agonist or it may be part of the LPS other than lipid A that has different sites of action on the target cells thus promoting further polarisation. When lipid A was tested in the polarisation assay, the response was rapid (Figure 8) similar to that of FMLP (Haston and Shields, 1985; Shields and Haston, 1985). The purity of the LPS samples should be considered as a possibility that may influence the biological activity. LPS samples from Dr. D.A.R. Simmons are known to be quite pure, and particularly are free of peptides and any proteins. However, the purity of commercially available LPS and lipid A are unknown.

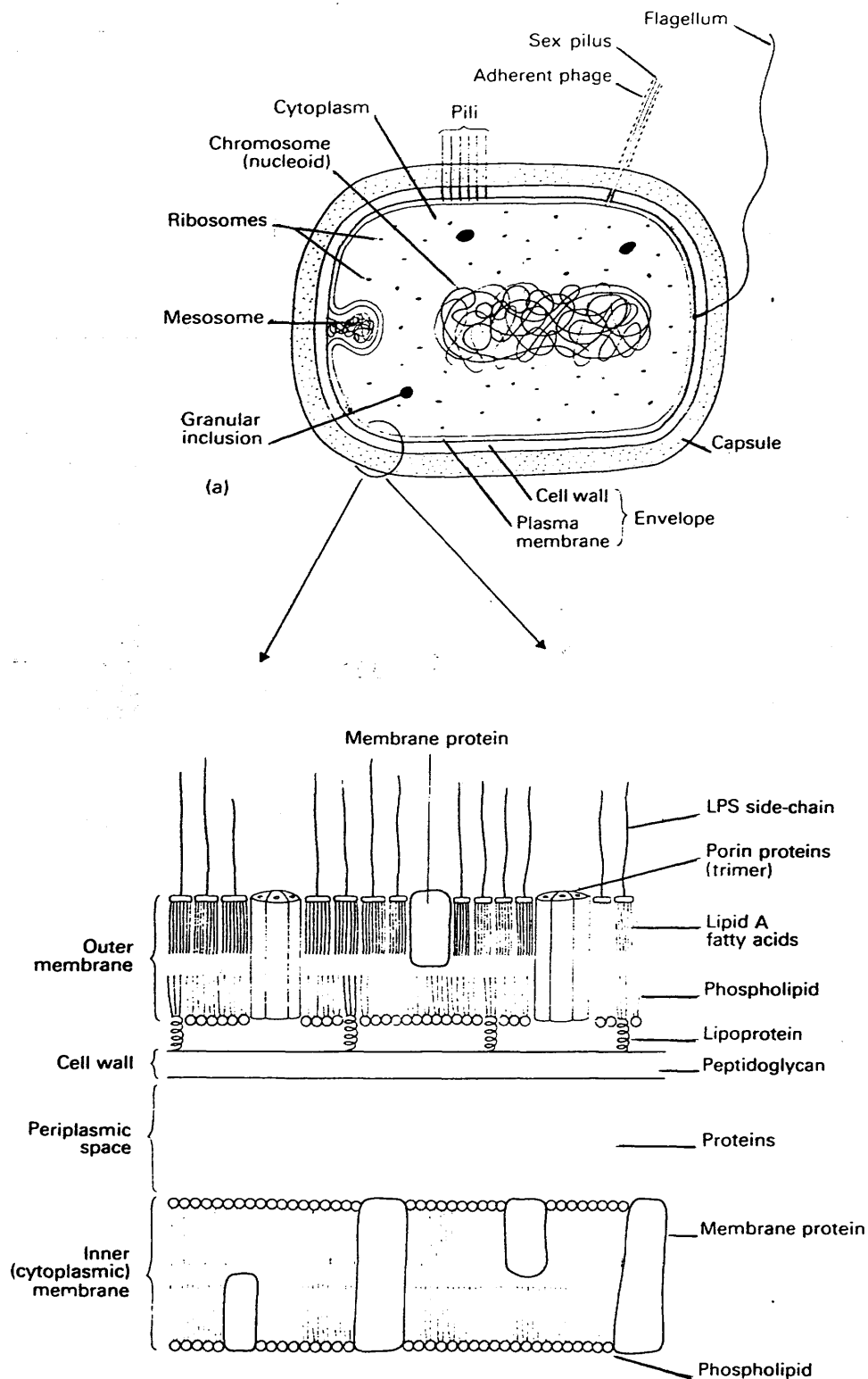


Figure 1. Schematic presentation of a Gram-negative bacteria and a detailed structure of the cell envelope. (Taken from *Processes in Pathology and Microbiology*, ed. Taussig, M.J. 1984)

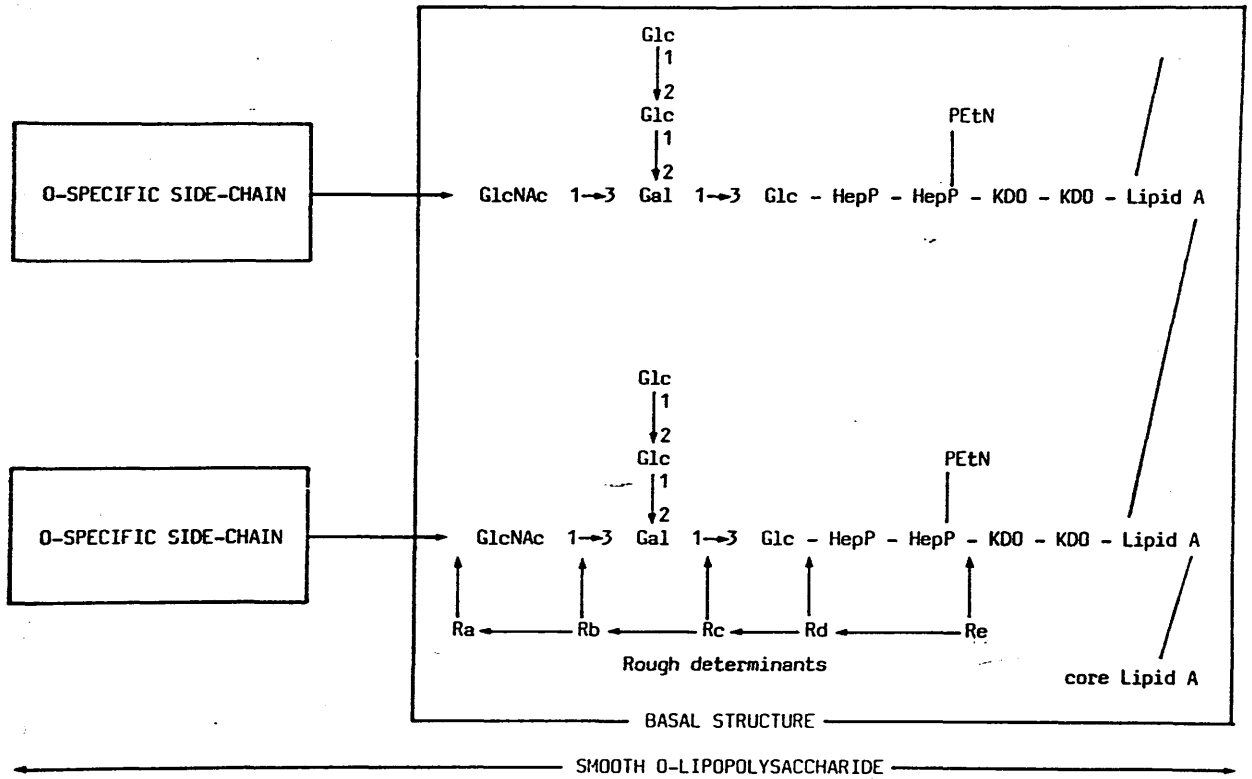


Figure 2. Diagrammatic presentation of the chemotypic position of a representative Gram-negative bacterial cell wall (*Shigella flexneri*). Reprint given by Dr. D.A.R. Simmons, Department of Bacteriology and Immunology, Glasgow University.

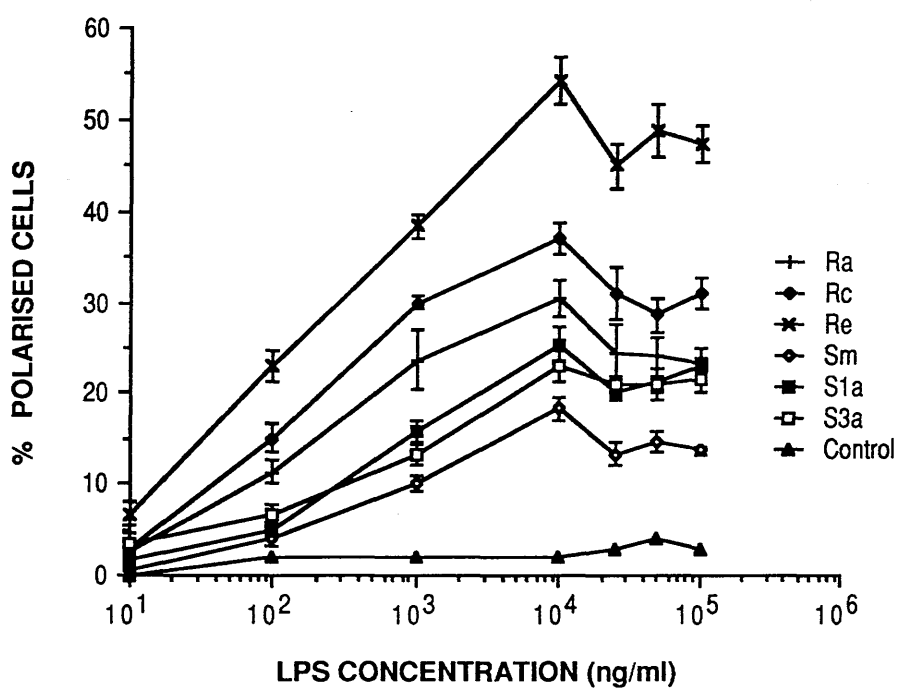


Figure 3. Dose response curve. Neutrophil polarisation in response to LPS from Shigella flexneri (Ra, Rc, S1a, S3a) and Salmonella minnesota (Re and Sm) at 37^o C for 60 minutes. Designation R represents rough strain while S indicates smooth strain. Bars indicate standard deviation values. (n=5)

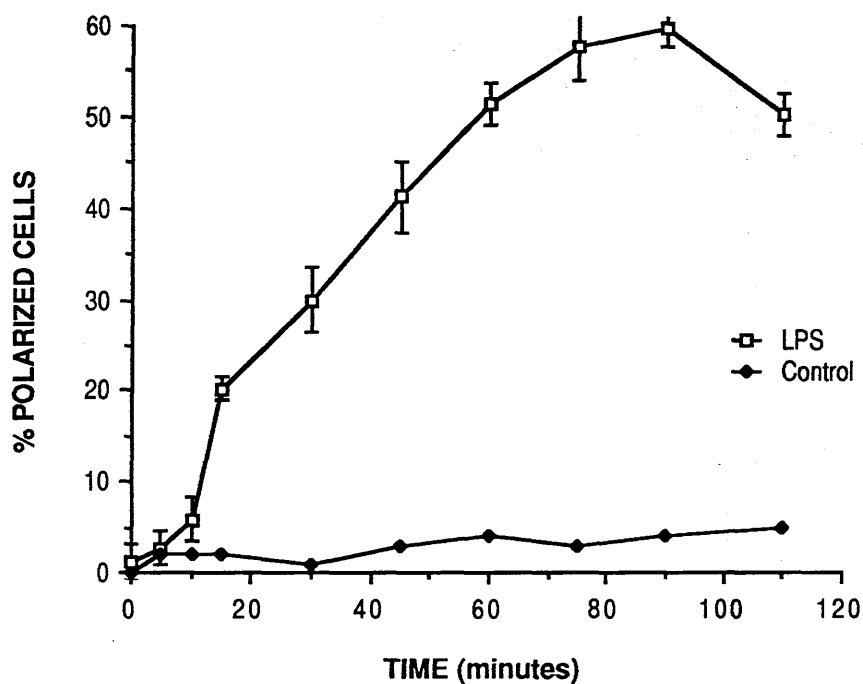
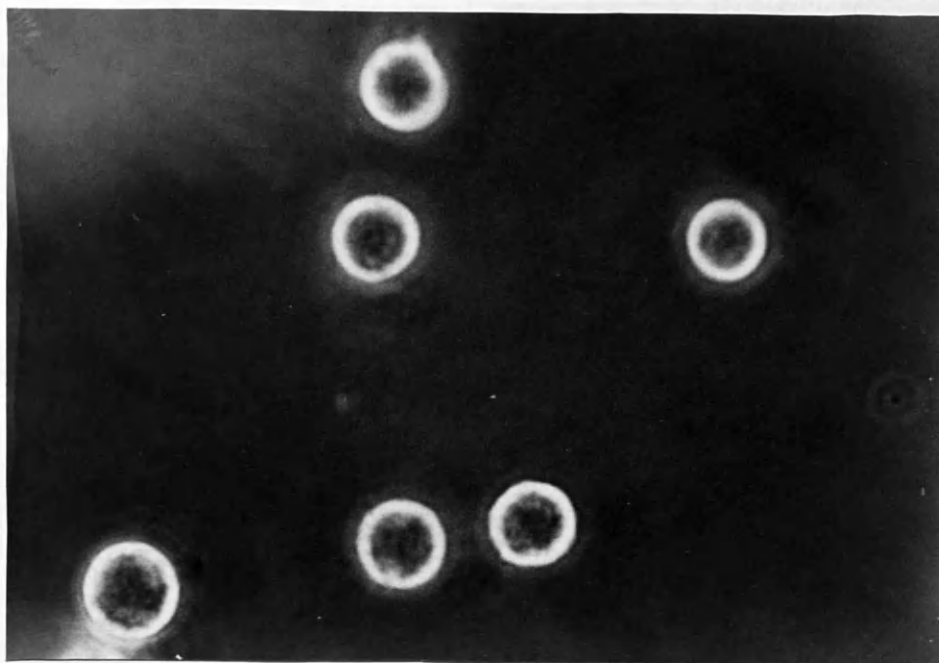
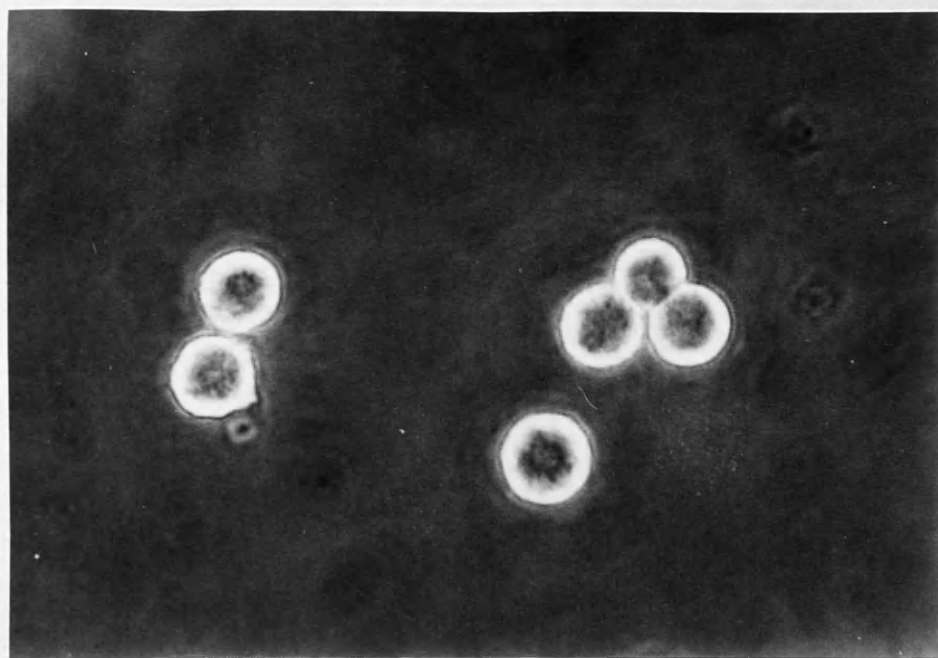


Figure 4. Time course of polarisation of neutrophils in response to LPS of mutant strain Salmonella minnesota Re 595 (10 ug/ml) incubated at 37° C. The values were obtained from 5 different experiments with the cell concentration of 10^6 cells/ml. Bars mean standard deviations.



(A) Straight from blood



(B) 5 minutes

Figure 5. Photographic sequence of cells polarising in LPS. Neutrophils were incubated with 10 $\mu\text{g/ml}$ LPS at different times and then fixed. The fixed cells were photographed and later printed. (X40 magnifications)



(C) 15 minutes

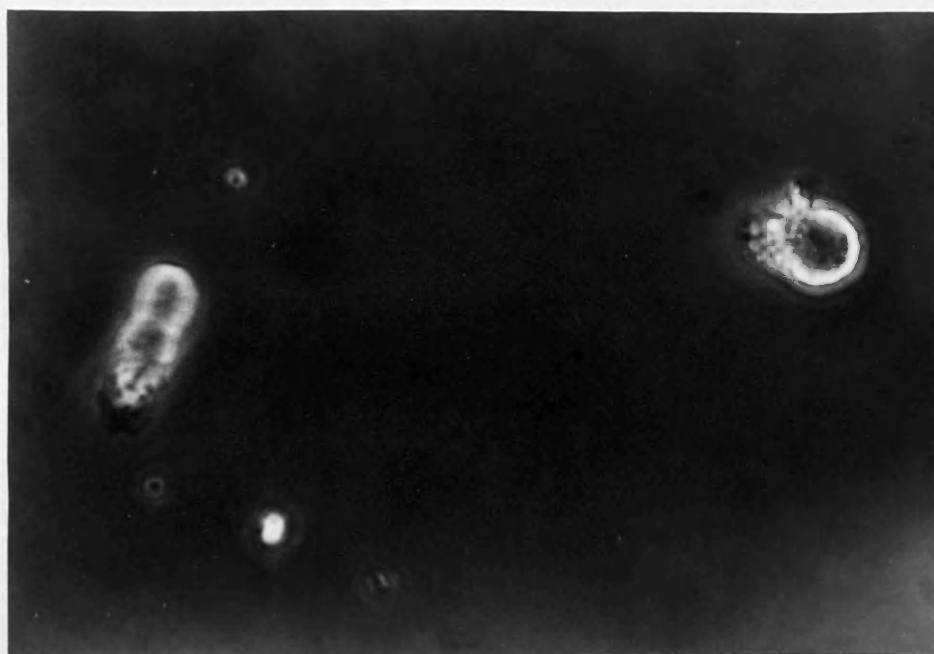


(D) 30 minutes

107g



(E) 45 minutes

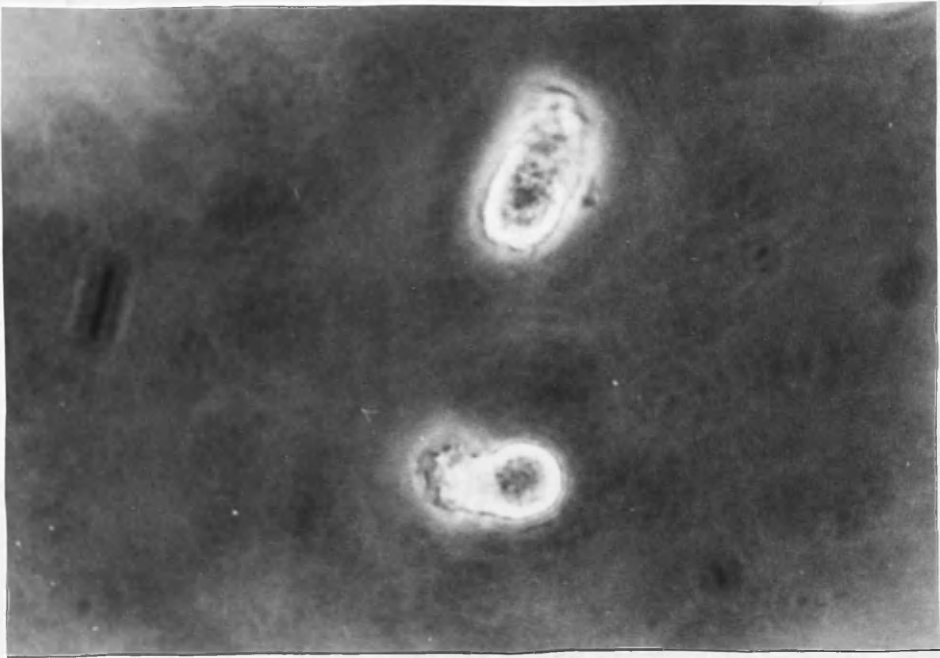


(F) 60 minutes

107h



(G) 90 minutes



(H) 120 minutes

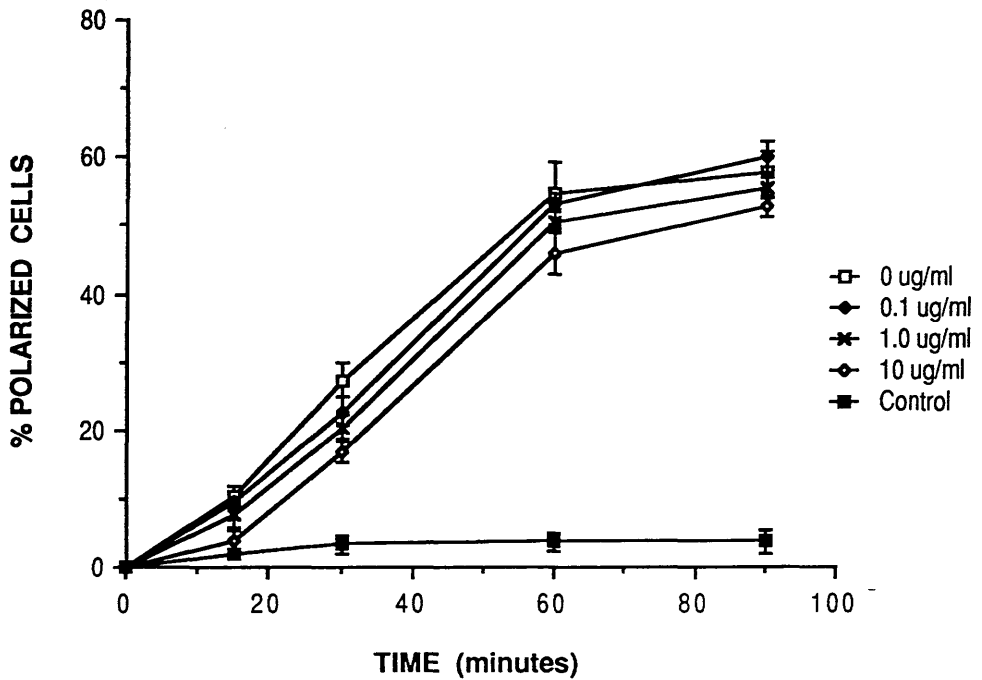


Figure 6. Effect of polymyxin on LPS-induced neutrophil polarisation using various polymyxin dosages. Neutrophil suspensions (10^6 cells/ml) were preincubated with polymyxin for 10 minutes at 37°C before challenging with LPS (10 ug/ml). The graph is based upon results from 3 different experiments with standard deviation bars.

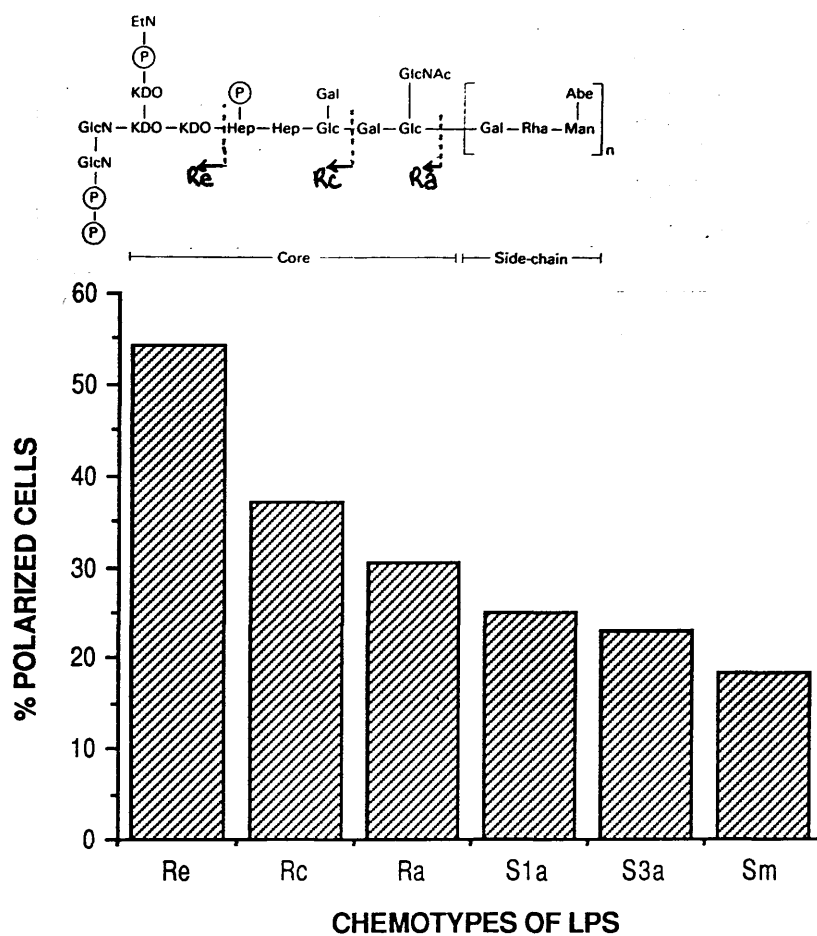


Figure 7. Relationship of the chemotypes of LPS (10^4 ng/ml) to the polarisation of the neutrophils. The time taken was at 60 minutes. The different chemotypes were from Salmonella minnesota strains (Re and Sm) and Shigella flexneri strains (Rc, Ra, S1a, and S3a).

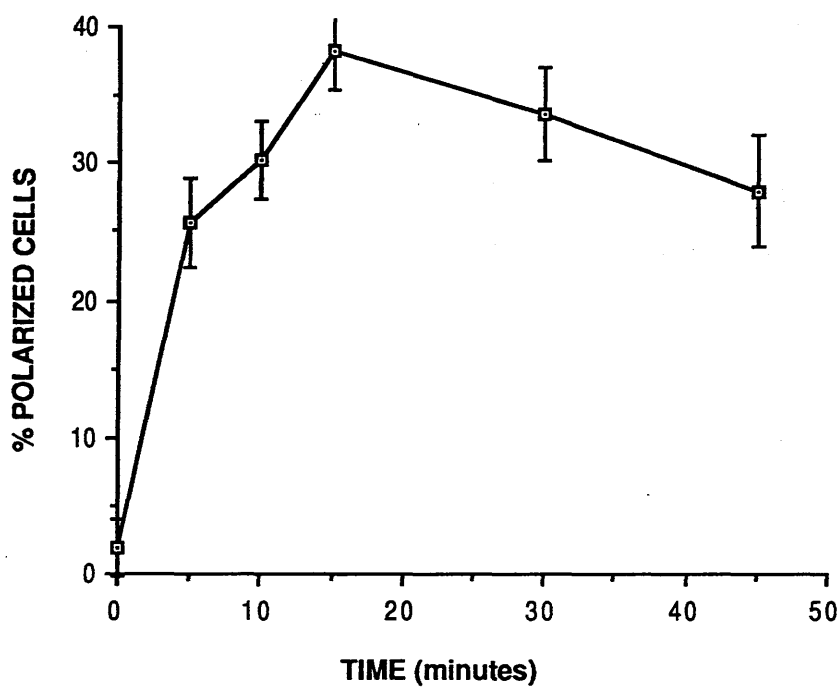


Figure 8. Time course of polarisation of neutrophils in response to lipid A of Salmonella minnesota (10 ug/ml) incubated at 37⁰ C. The values were from 3 different experiments with a cell concentration of 10⁶ cells/ml. The bars stand for standard deviation values.

Section 2

CELL-RELEASED FACTOR

The release by neutrophils of chemotactic factors which act on other neutrophils to cause phagocytosis and degranulation was investigated by several workers in response to a variety of stimuli (Phelps, 1969; Zigmond and Hirsch, 1973; Bentwood and Henson, 1980). The effect of LPS in inducing similar release of a cell-derived factor was studied by incubating neutrophils with 10 $\mu\text{g/ml}$ of *Salmonella minnesota* Re 595 LPS for 90 minutes, removing the supernatant and reincubating this fluid portion with a fresh batch of cells. Figure 9 shows that the response of the latter cells in assuming non-spherical shape was rapid (within 5 minutes of incubation) which is unlike the response to LPS alone and the maximum percentage of polarised cells, 29.5%, was attained after 10 minutes of incubation. Beyond this time period, the percentage of cells changing shape declined at first but after 30 minutes the number of polarised cells began to increase again slowly. The second phase assumed a similar pattern to that of polarisation using LPS directly. The supernatant collected was diluted X10 and X100 and then incubated with a new batch of cells for 10 minutes to obtain the maximal polarising activity. As the concentration of

supernatant decreased, the percentage of polarised cells was simultaneously reduced (Figure 10). The effect of cell density during the priming phase with LPS upon release of a supernatant factor was also studied by adding such supernatant to a fresh batch of neutrophils. As can be seen in figure 11, the lower the number of cells used to harvest the factor, the less the activity of the factor based on the results of the short-term polarisation assay. This suggests release of a cellular product whose total activity is dependent on cell concentration.

Discussion

Induction of polarization of neutrophils by LPS follows a time-course that is not typical of chemotactic factors. The latter produce polarization within 5 to 15 minutes (Shields and Haston, 1985) whereas LPS works very slowly. Therefore the expectation is that a chemotactic-type factor may have been released by cells on exposure to LPS. Supernatants prepared by pre-incubating neutrophils with LPS were shown to cause a rapid shape change in fresh neutrophils within 5 minutes compared to the longer time taken when cells were exposed to LPS directly. The indication then is that

after a certain period of exposure to LPS, the cells started to
release a factor detectable in sufficient quantity to cause
the leukocytes to exhibit polarised morphology. On diluting

out the supernatant, fewer cells were polarized. To support the view of the role played by the released factor further, cell density was varied to manipulate the amount of cell-released factor. A significant reduction was observed in the polarisation of PMN when cell concentration was 10^4 cells/ml in comparison to that of 10^6 cells/ml, an indication of lesser amount of factor produced. It is possible that polymyxin (section 1 of the results and discussion) was unable to cause any reduction in total cell shape change at the later stage of incubation partially due to the increased presence of a new agonist, insensitive to polymyxin, that induced non-spherical morphology.

An observation that needs further attention during the time course in short-term polarisation was the change in the percentage of cells polarising after 30 minutes. Instead of declining or levelling, the graph shows a slow increment in the percent polarisation. A possible explanation for this effect was the presence of free LPS in the supernatant. Polymyxin B sulfate which was again preincubated with PMN before challenging the cells with the supernatant was observed to result in a small reduction in the number of polarised cells (results not depicted graphically). Thuseven with a small amount of LPS as had been documented earlier, cells would still respond towards its presence amounting to the increase of polarised cell even though the effect is

not comparable to that of direct incubation of cells to LPS. The neutralising action of polymyxin on LPS consequently seemed to be relatively ineffective at longer time period as evident by the small difference in short-term polarisation assay of supernatant to that system with polymyxin present.

The degree of polarisation of neutrophils in response to LPS was compared with polarisation using LPS which had been neutralised with polymyxin. The results showed that the degree of polarisation was significantly reduced when LPS was neutralised with polymyxin. This suggests that the degree of polarisation is dependent on the amount of LPS present and that the degree of polarisation is not directly proportional to the amount of LPS present. The results also suggest that the degree of polarisation is not directly proportional to the amount of LPS present.

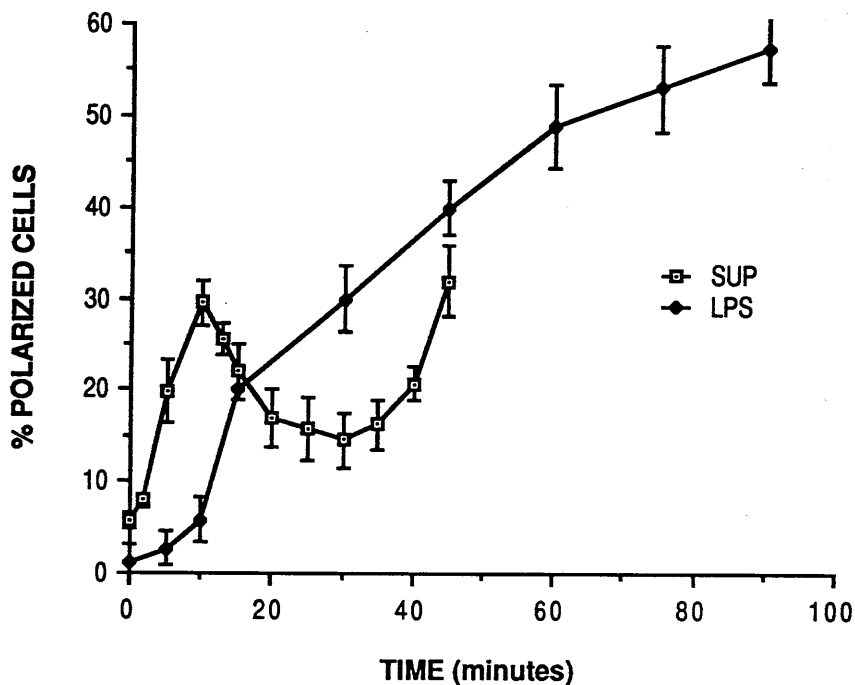


Figure 9. Time course of polarisation of neutrophils in supernatant in comparison with polarisation using LPS directly. The supernatant was obtained from preincubating PMN (10^6 cells/ml) in LPS (10 μ g/ml) for 90 minutes at 37° C. New batch of PMN (10^6 cells/ml) was added to tubes containing the supernatant or fresh LPS samples and incubated at 37° C. Five different experiments were used to plot the graph. The bars stand for standard deviation values.

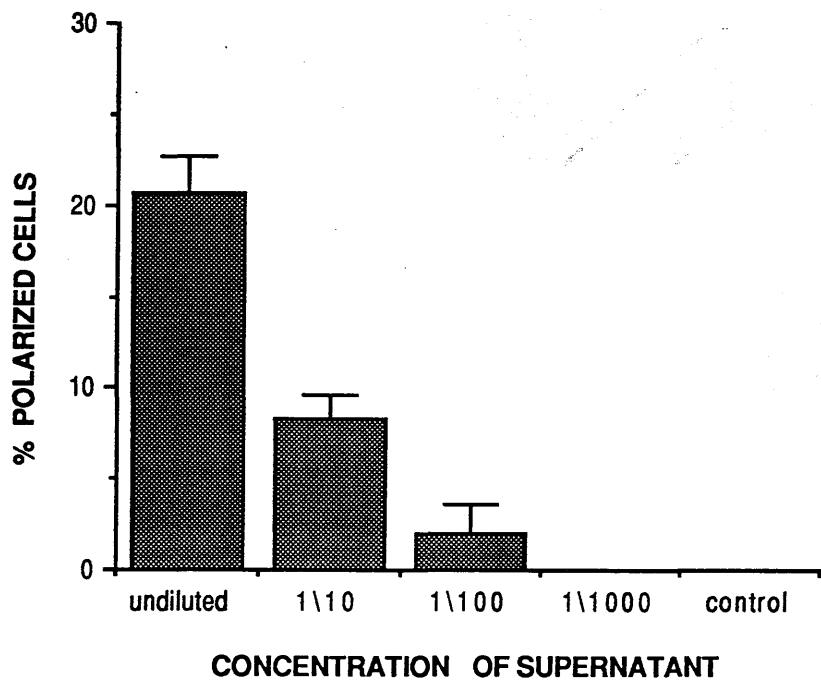


Figure10. The effect of diluting supernatant in short term polarisation assay. Supernatant was recovered from preincubating neutrophils (10^6 cells/ml) in 10 ug/ml LPS at 37° C for 90 minutes. New batch of cells was then added to the supernatant and incubated at 37° C for 10 minutes for polarisation assay. The data were taken from 3 different experiments with standard deviation values represented by the bars.

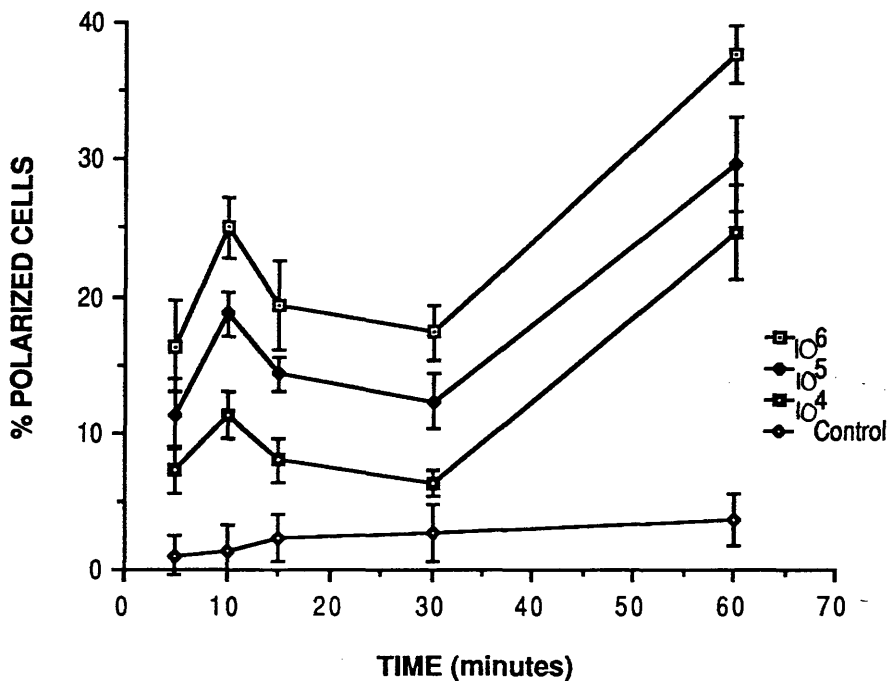


Figure 11. The effect of cell density on the release of polarising factor. Three different concentrations of neutrophils (10^6 , 10^5 , and 10^4 cells/ml) were initially preincubated with 10 ug/ml of LPS for 90 minute at 37°C after which the supernatants were saved and tested on new batch of neutrophils (10^6 cells/ml) in polarisation assay at 37°C . The data presented were from 5 experiments. Bars are standard deviation values.

Section 3

MICROPORE FILTER ASSAY

Testing the supernatant in short-term polarisation assay does not directly test the locomotory ability of the cells promoted by the cell-released factor. Another approach was used to test that the factor released by the neutrophils in the presence of LPS not only induces shape change but also induces locomotion and chemotaxis. The micropore filter assay was used for this purpose.

To test if the supernatant collected after incubating PMN at 10^6 cells/ml with $10\text{ }\mu\text{g/ml}$ of LPS for 90 minutes contained chemotactic activity as well as the capacity for stimulation of cell locomotion, different concentrations of the supernatant were placed above and below the filters. Both absolute concentration and the concentration gradient were varied using a checkerboard assay (Zigmond and Hirsch, 1973) as shown in table 2 in which migration towards a given substance at different concentrations was tested by placing the cells in chambers of positive gradients, negative gradients or in the absence of a gradient. In table 2, cells in the chambers along the diagonal from the upper left position to the lower right were migrating in the absence of any concentration gradient. Moving down the diagonal the absolute concentration of the supernatant to which the

cells were exposed increased and so did the migrated distance. Raising the concentration of the attractant influenced the cell migration to a certain extent reaching a maximum mean of $86.1\ \mu\text{m}$ before eventually decreasing. Above the diagonal from the upper left to lower right the cells were migrating from a low concentration to a higher concentration of the supernatant, a situation of positive gradient. Below this diagonal they are moving through a high concentration to a lower one, a negative gradient. The experimental values above the diagonal are in general higher than the corresponding values on and below it, an outcome that would be expected to have resulted from a chemotactic effect of the supernatant but not a confirmatory evidence. Confirmation on this effect could be derived by determining the migrated distance of the cells assuming that they responded only to the various absolute concentrations and not to the concentration differences. It is then necessary to compare the expected or calculated values with that of the observed experimental values. In order to achieve this confirmatory test mathematical calculations outlined in the paper of Zigmond and Hirsch (1973) were followed using a programmable computer and diskette to obtain the values.

Cells migrating in the positive gradients had higher experimental figures (without parentheses) than those calculated (in parentheses) while neutrophils locomoting

in negative gradients had higher calculated values than that of the experimental origin. These differences between the two figures, calculated and experimental, demonstrate the response of the cells towards the absolute concentrations as well as the chemotactic response to the concentration gradients of the attractant in the supernatant.

Discussion

Shape changes of inflammatory cells induced by agonists are not always associated with locomotion (Keller and Zimmermann, 1987; Wilkinson et al, 1988). Methods to analyse leukocyte locomotion directly are needed. The micropore filter assay has enabled cell biologists to differentiate motile behaviour of PMN towards substances that may be chemotactic or otherwise. If cells migrate from zero concentration of a chemotactic substance to a higher concentration, this is not alone a sufficient proof of chemotaxis.

These cells might just be responding towards the ambient absolute concentration of attractant. The checkerboard assay was therefore adopted to distinguish chemotaxis from chemokinesis. Comparison between the calculated values and the experimental values suggested that the supernatant had exerted chemotactic influence; in general cells moved further in a positive gradient

and less far in a negative gradient. It has been shown that the calculations employed in the checkerboard assay do approximate to a real result that can be obtained in the absence of any chemotactic effects (Wilkinson and Allan, 1978). The supernatant in question thus fulfilled the criteria required of a chemotactic factor as analysed by the checkerboard system.

		Percentage of supernatant below filter				
		0	10	30	70	100
Percentage of supernatant above filter	0	57.5 ± 3.70	79.9 ± 3.61 (61)	83.6 ± 3.58 (67)	95.0 ± 2.90 (73)	86.9 ± 2.88 (76)
	10		73.2 ± 1.47	81.6 ± 2.12 (75)	90.1 ± 1.92 (78)	97.3 ± 2.00 (80)
	30		68.5 ± 3.26 (78)	79.7 ± 2.69	87.3 ± 2.61 (81)	91.2 ± 2.82 (83)
	70		66.1 ± 3.56 (83)	74.2 ± 3.28 (84)	86.1 ± 2.98	84.7 ± 3.80 (85)
	100		64.3 ± 4.58 (84)	70.7 ± 3.13 (84)	80.1 ± 3.11 (82)	81.1 ± 2.21

Table 2. Migration of neutrophils in concentration gradients of supernatant in micropore filter assay at 37° C for 60 minutes (measuring units in μm). Values in brackets represent the calculated values with $n=5$. The observed values are shown as distance of migration \pm standard deviation values.

Section 4

COLLAGEN GEL ASSAY

Another assay that can provide a better insight into cell locomotion is the collagen gel assay. The three-dimensional matrices of the gel give a much closer physiological setting for cell locomotion than the filter assay. Figure 12 shows a time course for invasion of collagen gels by the neutrophils in the presence and absence of the LPS-induced supernatant factor following the methodology outlined by Islam et al (1985). The cells in a uniform concentration of the supernatant responded well and migrated deep into the gels in comparison to cells in a gradient of the supernatant. The former condition was achieved by overlaying neutrophils in supernatant on top of collagen gel incorporated with similar concentration of supernatant present in the cell suspension. For the latter condition, collagen gel was impregnated with the supernatant and later overlaid by another portion of gel without the supernatant so as to form a gradient source. A suspension of neutrophils was layered on top of the second gel layer to allow the cells migrating in response to the gradient. As the time allowed to invade the gels increased, the distance travelled increased eventhough not in a linear fashion. Over three hours of invasion, the depth of the migration increased very

slowly. Distinct morphological characteristics were noted for the migrated cells in contrast to that of the initial preparative stage of the cells on top of the gel (Figure 13). Extensive polarised morphology was assumed as the cells penetrated deep into the gel.

Discussion

Collagen is a more physiological substratum than glass or tissue culture plates thus making it an ideal choice for the study of cell locomotive behaviour. Besides using the micropore filter assay to investigate if the supernatant did have the chemotactic factor, a similar kind of assay was used in the collagen gel assay.

The displacement of the cells in gradients was lower than in the chemokinetic condition (in uniform concentrations). This difference might be because in B (Figure 12) a lower concentration of the supernatant reached the cells. The behaviour of neutrophils in collagen is not fully understood but the medium permits clear morphological changes to be observed when a single cell moves through a physiological environment mimicking that of a normal tissue.

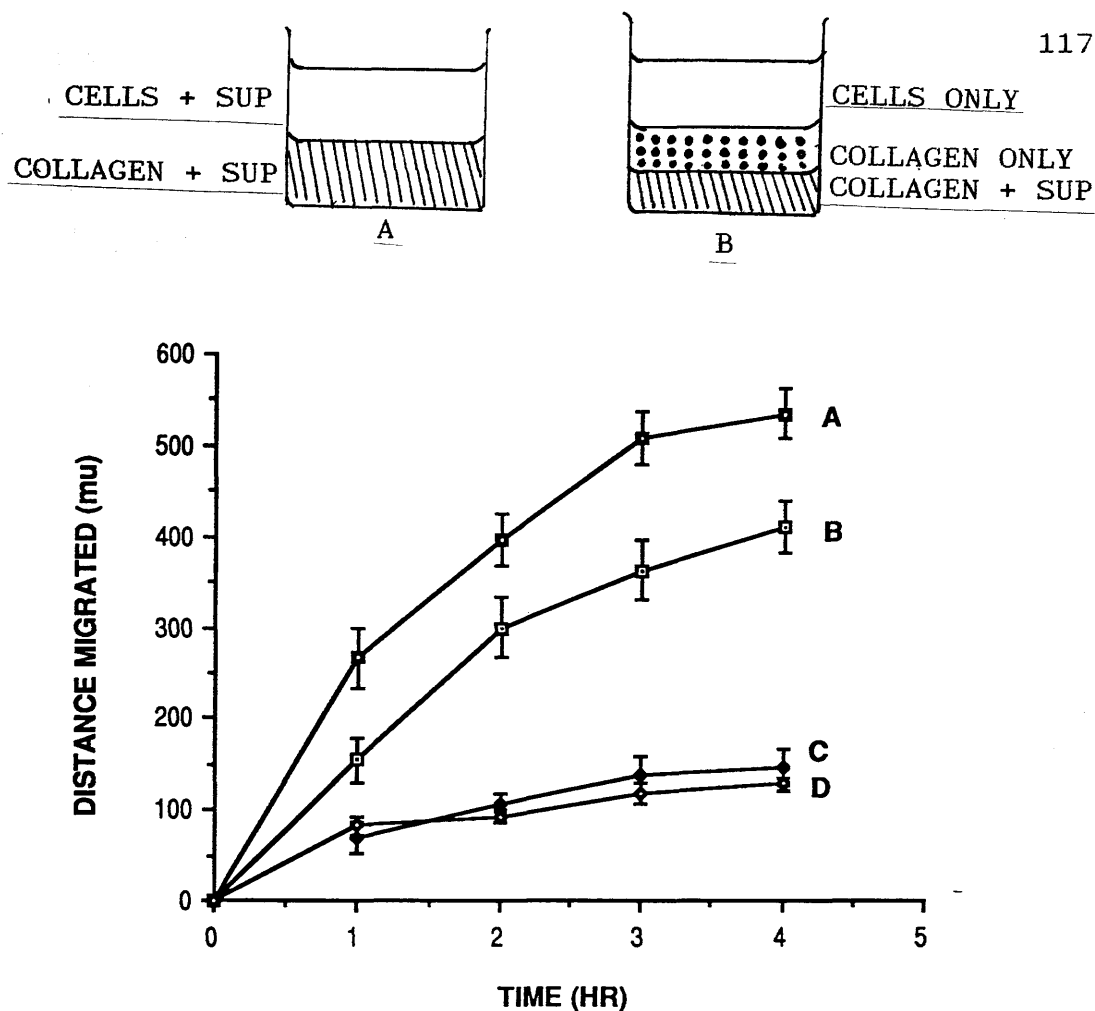
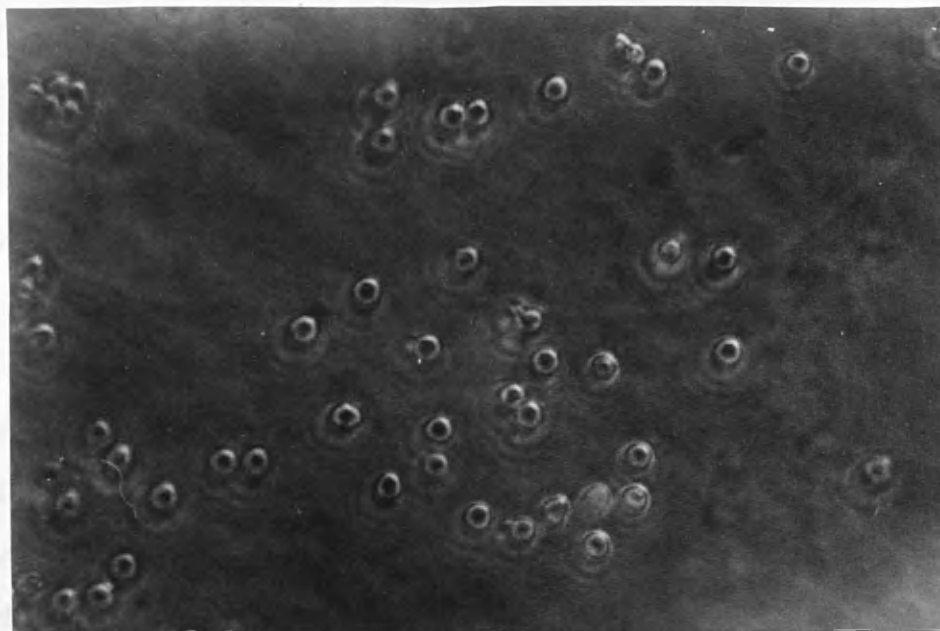
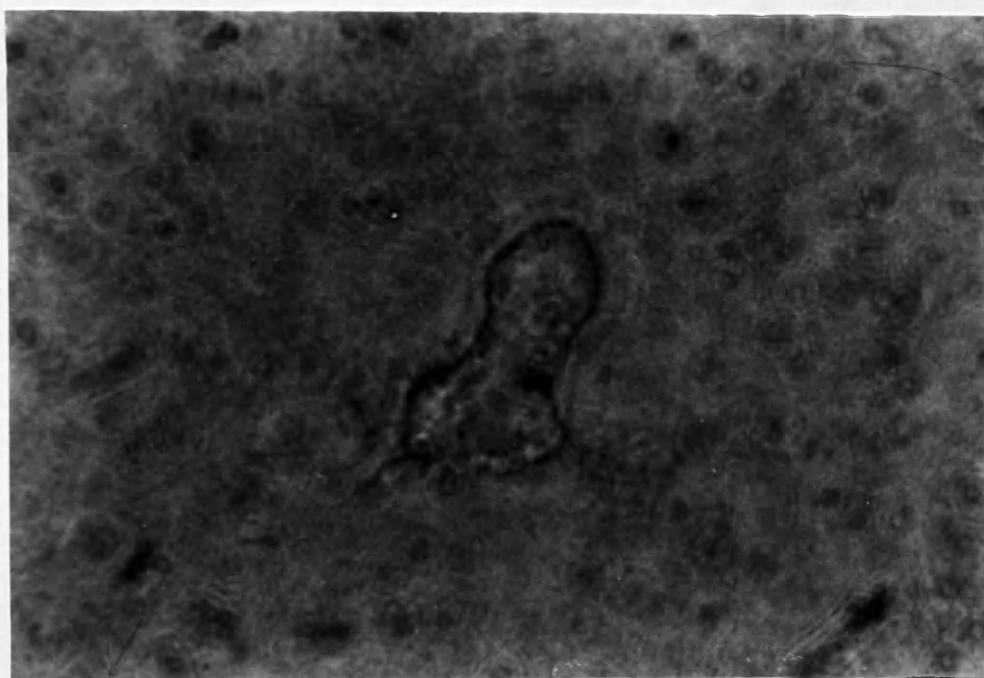


Figure 12. Collagen gel assay. PMN at 10^6 cells/ml was layered on top of the gel in the presence of or without supernatant at 37°C . The supernatant was obtained by preincubating PMN (10^6 cells/ml) with LPS (10 $\mu\text{g/ml}$) for 90 minutes at 37°C . Data plotted were from 5 different experiments. Bars are standard deviations of the means. Results from condition A are expressed graphically as curve A with the control as curve D. Results from condition B (2 layers of collagen with a gradient through the top layer) are represented by curve B with the control as curve C.



(A)



(B)

Figure 13. Photographs of PMN in collagen assay. Picture A (X10 magnification) shows cells on top of the gel while picture B (X100 magnification) focuses on cells migrating inside the gel.

Section 5

VISUAL STUDIES

The polarisation assay measures deviation from the spherical to non-spherical shape at one point in time. The actual movements of the cells are not observed. A visual assay using a video camera helps to give more detail of the actual behavioural response of these cells and measuring morphological parameters of the activated cells is an additional method to quantify the degree of shape change.

Shape change in suspension

Neutrophils that were preincubated with or without LPS for various times were quickly transferred to a filming chamber and their movements were videotaped. Individual cells were singled out to record the shape changes in suspension for different time periods of LPS exposure when the videotape was played back. The tracings of the shape changes in suspension of the neutrophils show that the front-tail polarity of individual cells did not remain constant in direction, but changed with time (Figure 14). The cells frequently elongated and then rounded up before eventually settling down on the slide. Greater number of movements were quite obvious after the cells have being exposed to the LPS for over 30 minutes with a faster speed of changing

shapes. The observations indicated that the cells were activated by an agonist since the majority of these cells remained spherical when observed in the absence of a stimulus.

Shape analysis

To define shape change induced by the presence of LPS more precisely, analytical assessment was done following the method of Dunn and Brown (1986). Neutrophils, LPS-induced and FMLP-induced, polarising at different time periods were fixed and the outlines traced before analysing them with a computerised programme. All cells with a dispersion value greater than or equal to 0.02 were selected as non-spherical for further calculations (Wilkinson et al, 1988). Figure 15 shows representative samples of the shapes incubated at different times with LPS or FMLP. Tracings of the shapes of cells in LPS displayed a progression in the morphological changes from the start of the incubation period up to 90 minutes. These cells were much more elongated and all of the selected cells had a dispersion value of greater than 0.02 after 90 minutes. Cells in FMLP had acquired a high elongation value even after 30 minutes though the biggest median value for elongation was after the 90 minutes mark. The deviation from spherical morphology as measured by the dispersion value was however evident for the total cell samples as early

as 10 minutes (Table 3). Using Mann-Whitney U-tests, the difference in shape change within the LPS-induced group was evaluated (Table 4). Statistical analysis was also done to compare if the shape change induced by LPS was similar to that of the classical morphological change due to FMLP. Significant differences were observed between the groups (Table 4). Up to 60 minutes FMLP induced greater shape-change than LPS. However, at 60 minutes and 90 minutes no significant difference was found in the shape change between these two groups.

Discussion

Studying shape change induced by a stimulant by a visual method proved to be valuable in distinguishing between the classical shape change due to a known chemotactic stimulus and that of a non-specific inducer such as phorbol esters (Wilkinson et al, 1988). Videotaping shape changes in suspension gave much more information about the detailed activity of the cells than studying fixed cells in polarisation assay. PMN were observed to continuously and vigorously change in shape after a longer period of time exposed to LPS before they finally settled down on to the surfaces. Even though broad veils protruding were not well presented, protrusions and retractions of cellular membrane from more than a single point were vividly recognised. Constrictions of the cell body were demonstrated by the

motile cells. Rapid morphological changes noted at later times may be related to the presence of a second agonist that seemed to be responsible for the locomotory behaviour since in the absence of LPS the cells frequently assumed rounded shapes with small shape changes in suspension.

Analysing the shape change using shape parameters (elongation, extension and dispersion) allowed a comparison of the LPS-induced polarisation with that due to FMLP. Long exposures (60 to 90 minutes) showed no significant difference in elongation from the results for FMLP. This is consistent with the presence of an agonist that induced a similar effect to that of the known chemotactic factor FMLP. The significant difference between the two stimuli at earlier stages (up to 60 minutes) may be due to the fact that LPS has no direct effect in polarizing cells but causes delayed release of a cell-polarizing factor. During this preparative period those cells that were able to respond well were therefore activated by LPS but had not yet released the polarizing factor.

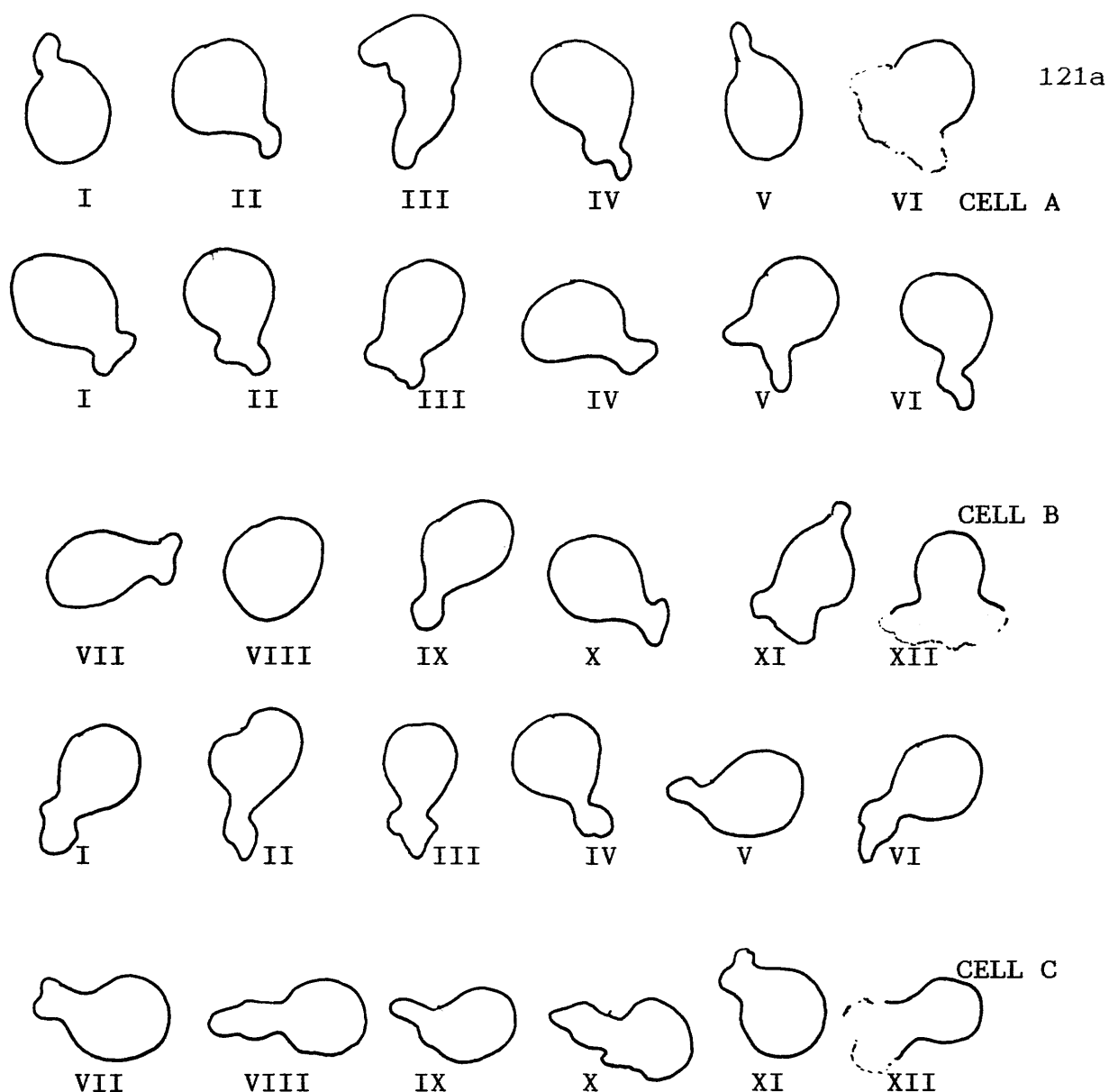
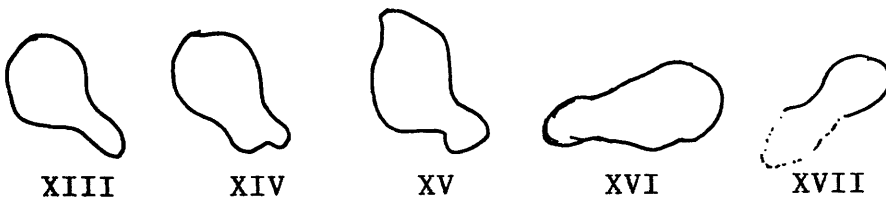
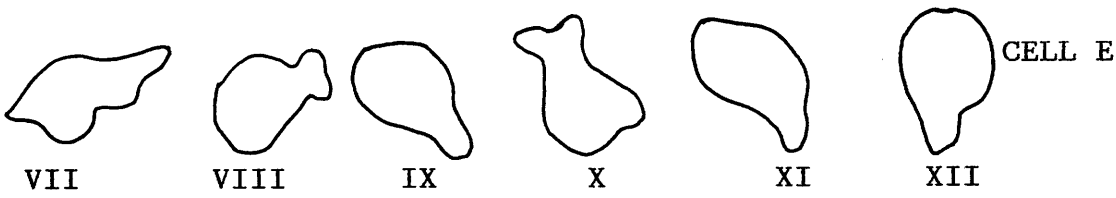
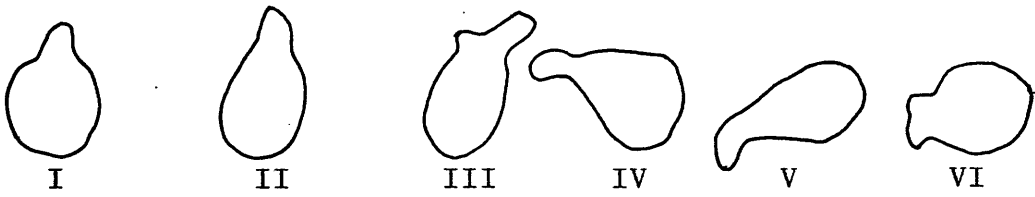
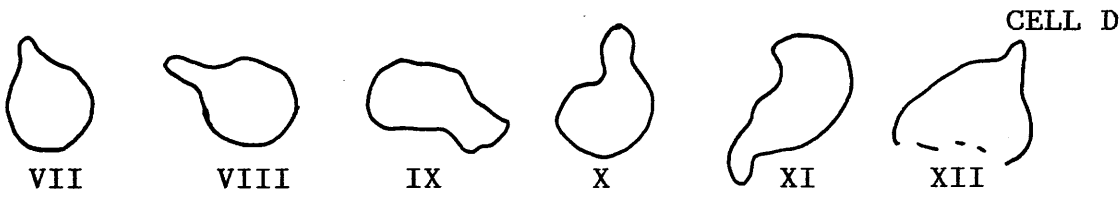
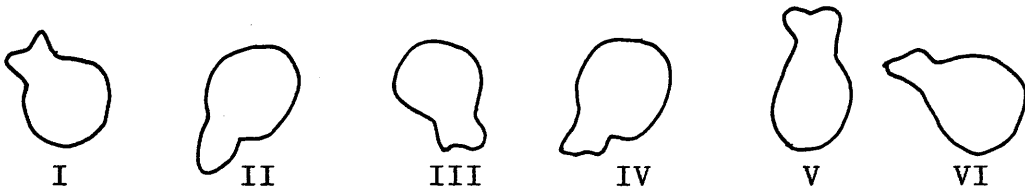


Figure 14. Live cells using videotape. PMN incubated at different times with LPS were observed in filming chamber and videotaped. Motions of the cells in suspension were traced out at 30 seconds intervals (Roman numerals). Times of incubation: Cell A- 30 min, Cell B- 45 min, Cell C- 60 min, Cell D- 90 min, Cell E- 120 min.



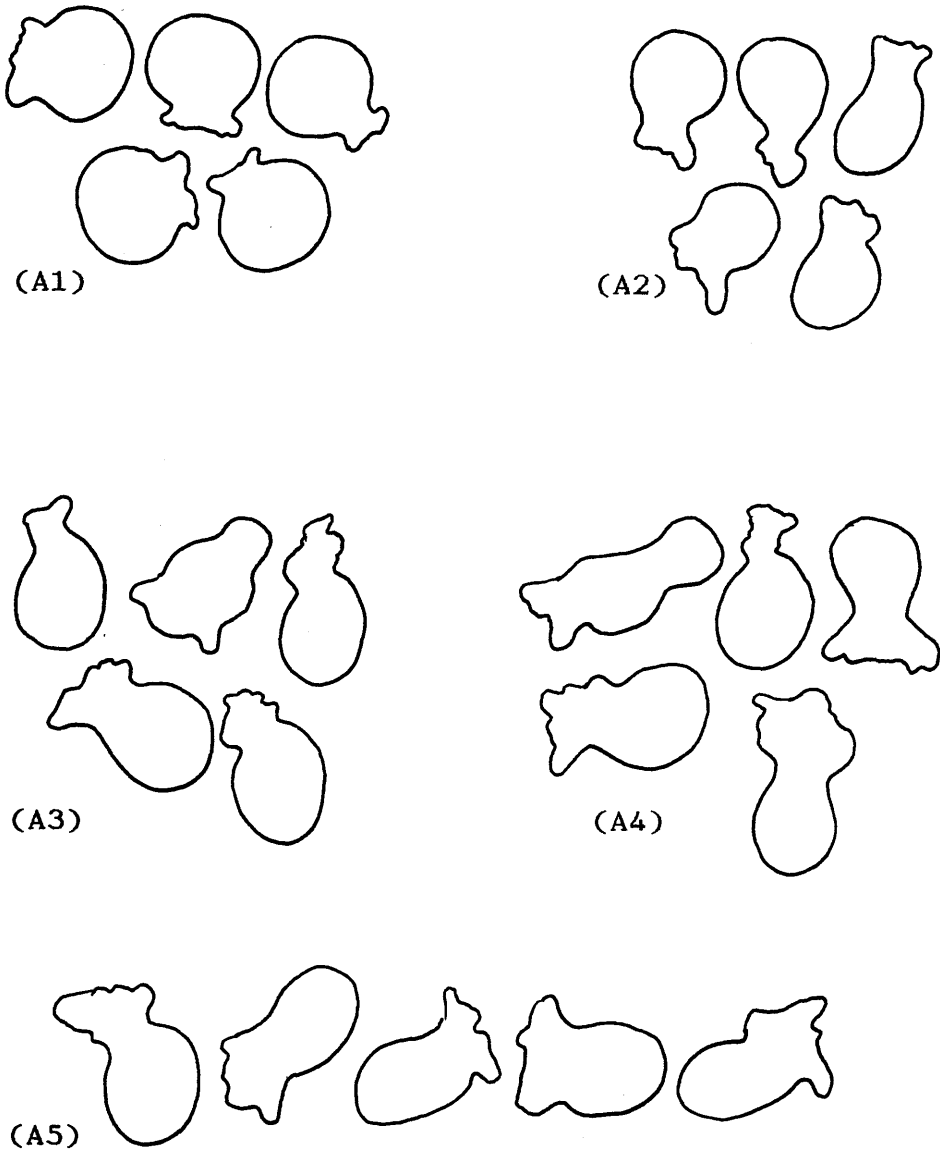
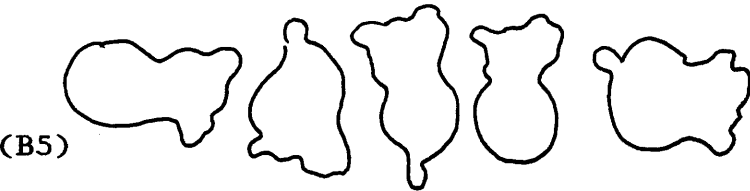
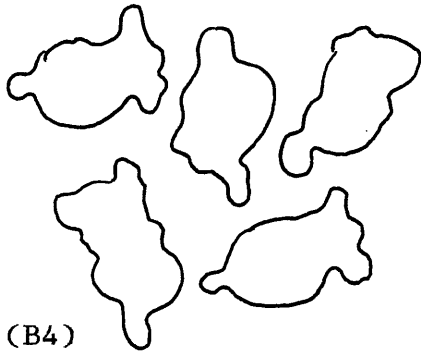
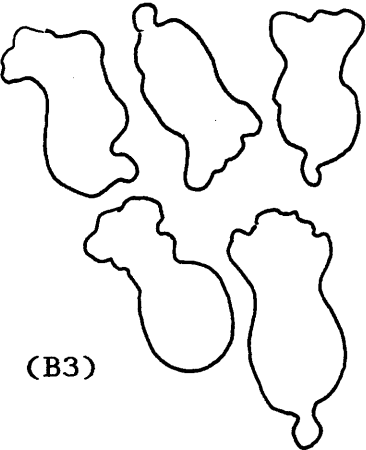
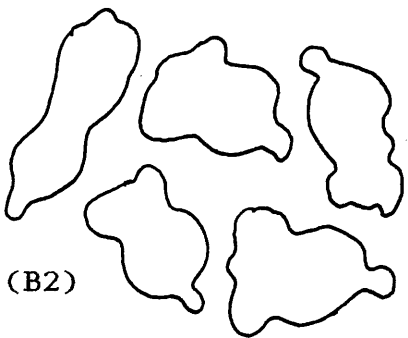
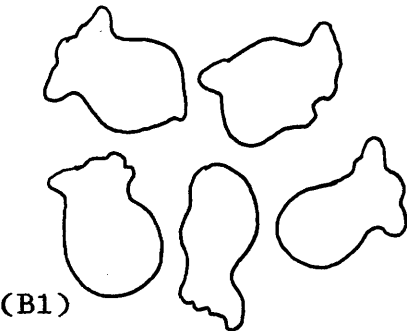


Figure 15. Fixed cells using camera lucida. Representative samples of shape changes of PMN in the presence of LPS or FMLP at different periods of incubation were outlined after fixing them. Cells in LPS are shown as (A1) 10 min, (A2) 15 min, (A3) 30 min, (A4) 60 min and (A5) 90 min. PMN in FMLP are drawn as (B1) 10 min, (B2) 15 min, (B3) 30 min, (B4) 60 min and (B5) 90 min.



		Median values		
	n (%)	Extension	Dispersion	Elongation
PMN in LPS for:				
0 min	1 (2)	-	-	-
10 min	16 (32)	0.394	0.034	0.365
15 min	23 (46)	0.666	0.071	0.612
30 min	44 (88)	0.653	0.060	0.577
60 min	49 (98)	0.864	0.098	0.744
90 min	50 (100)	0.874	0.092	0.755
PMN in FMLP for:				
10 min	49 (98)	0.730	0.069	0.658
15 min	50 (100)	0.737	0.063	0.673
30 min	50 (100)	0.919	0.073	0.843
60 min	50 (100)	0.866	0.087	0.782
90 min	50 (100)	0.920	0.076	0.851

Table 3. Measures of shapes of neutrophils. The cells were either incubated in LPS (10 $\mu\text{g/ml}$) or FMLP (10^{-8} M) at the stated times before being fixed and later statistically analysed using Mann-Whitney U-test. 'n' is the number of polarized cells (out of 50) (%) is the percentage of polarized cells Cells with dispersion < 0.02 were excluded.

Sample	Extension	Dispersion	Elongation	Significance
10 vs 60	4.85	5.43	4.29	P< 0.001
10 vs 90	5.11	5.15	4.56	P< 0.001
15 vs 60	4.41	5.34	4.18	P< 0.001
15 vs 90	4.59	4.89	4.26	P< 0.001
30 vs 60	3.88	4.12	3.32	P< 0.001
30 vs 90	3.93	3.63	3.45	P< 0.001
L10 vs F10	3.41	4.05	3.32	P< 0.001
L15 vs F15	4.50	3.40	4.58	P< 0.001
L30 vs F30	4.07	4.10	3.88	P< 0.001

Table 4. Statistical analysis of shape changes. Using Mann-Whitney U test difference of PMN morphological alterations after treatment with LPS or FMLP was analyzed. The first half of the table depicts comparison between each period of PMN incubations in LPS (sample= time versus time). The second half of the data compares shape changes between cells incubated in LPS (L) and those in FMLP (F). Unlisted are those which were insignificant in the shape change differences.

Section 6

EFFECT OF ANTI-INFLAMMATORY DRUGS

Anti-inflammatory drugs have been utilised to block the release of certain factors by inflammatory cells by interfering with arachidonic acid metabolic pathways. These drugs were tested on the polarisation system to determine if PMN shape change in the presence of LPS could be blocked and if so this may suggest that metabolites from the arachidonic acid pathway may be involved in the shape change of the cells. One steroidal (dexamethasone) and three non-steroidal (BW 755C, Revlon

5901A and indomethacin) anti-inflammatory drugs were tested. Dexamethasone indirectly, by stimulating synthesis and release of lipocortin, blocks the action of a membrane

bound enzyme called phospholipase A₂ needed to switch on arachidonic acid metabolic pathways (Flower and Blackwell, 1979). BW 755C is reported to possess the capacity of inhibiting the lipoxygenase and cyclooxygenase pathways in the cell membrane (Higgs et al, 1979). The former pathway is responsible for the release of leukotrienes while the latter leads to the production of prostaglandins. Revlon on the other hand is a specific lipoxygenase inhibitor thereby interfering with the release of the potent chemotactic agent leukotriene B₄ (Rossi, 1987), while indomethacin is a cyclooxygenase inhibitor (Lands, 1981).

Indomethacin, dexamethasone, BW 755C and Revlon 5901A at a concentration of 10 $\mu\text{g/ml}$ each were preincubated with 10^6 PMN/ml for 10 minutes at 37°C prior to exposing the cells to 10 $\mu\text{g/ml}$ of LPS. The stated dosage was chosen after three different doses (5, 10 and 15 $\mu\text{g/ml}$) were tried which resulted in a better inhibition at 10 $\mu\text{g/ml}$. The blockading effect was analysed between 15 to 90 minutes of incubation time. Factor production was eventually tested by preincubating the cells with the respective drugs for 10 minutes and then adding LPS, allowing incubation to proceed for another 90 minutes before finally saving the supernatant to be tested on the new batch of cells in short-term polarisation assays.

Indomethacin and dexamethasone were observed to have failed in preventing either the shape change or the production of the factor. However, BW 755C and Revlon 5901A were able to lessen the number of cells polarising significantly only at a 60 minutes time period (Figure 16). As for the factor release, a significant difference was seen for both drugs in comparison to that of the control system having no drugs (Figure 17) when supernatants were tested on a fresh batch of neutrophils. Excess NSAID in the supernatant may have a residual effect on the new batch of cells responding towards the factor in the medium. Therefore, the new batch of cells was initially preincubated with NSAID

before being challenged with the supernatant. Such experiments showed the absence of any reduction in the polarisation of the cells hence ruling out the effect of the drugs in inhibiting these cells from responding towards the unknown agonist (results not shown). All the drugs tested were found not to be cytotoxic at the concentration level used and did not by themselves activate the cells to assume the polarised shape.

Discussion

One of the ways by which PMN release chemotactic factors is via arachidonic acid metabolism. NSAID have been tried to block arachidonic acid metabolism and apparently reduce or stop the releasing of the chemotactic factors. Conventional drugs like indomethacin and dexamethasone do not reduce shape change activity but the new generation of NSAID exemplified by BW 755C and Revlon 5901A may work well in preventing the release of cellular factors. Both drugs were observed to caused a marked reduction of polarised cells after 60 minutes of incubation. The chemotactic type activity of the supernatant was consequently affected hence providing a partial clue to the possible involvement of arachidonic acid metabolites in this system.

Results from the in vitro and in vivo studies of NSAID on neutrophils have demonstrated that cellular

reponses such as lysosomal release induced by FMLP, concanavalin A and PMA were inhibited by the drugs (Abramson et al, 1984). Even though these findings implied that the modulation of neutrophil functions was dependent on leukotrienes synthesis inhibition, however there are diverse effects observed among the cyclooxygenase inhibitors in the inhibition of neutrophil functions such as inhibition of superoxide anion production (Kaplan et al, 1984; Minta and Williams, 1985). Similar drug effects were observed on the polarising capacity of neutrophils in the presence of LPS thereby indicating that anti-inflammatory effects of NSAID may not be attributed solely to their ability to inhibit prostaglandin synthesis but rather may extend to other metabolic effects on neutrophils such as leukotriene synthesis.

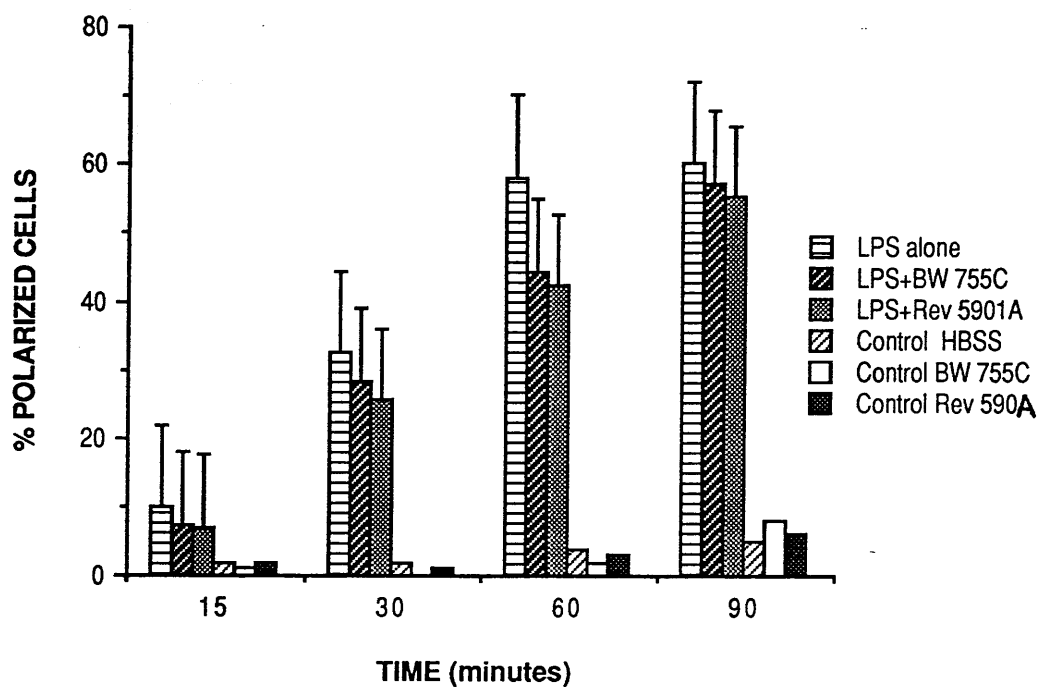


Figure 16. The effects of non-steroidal anti-inflammatory drugs on LPS-induced neutrophil polarisation assay. The cells at 10^6 cells/ml were exposed to the drugs (10 μ g/ml) for 10 minutes at 37 $^\circ$ C followed by the addition of LPS at 10 μ g/ml. Five experiments were done for each drug used. (Bars are standard deviation values.)

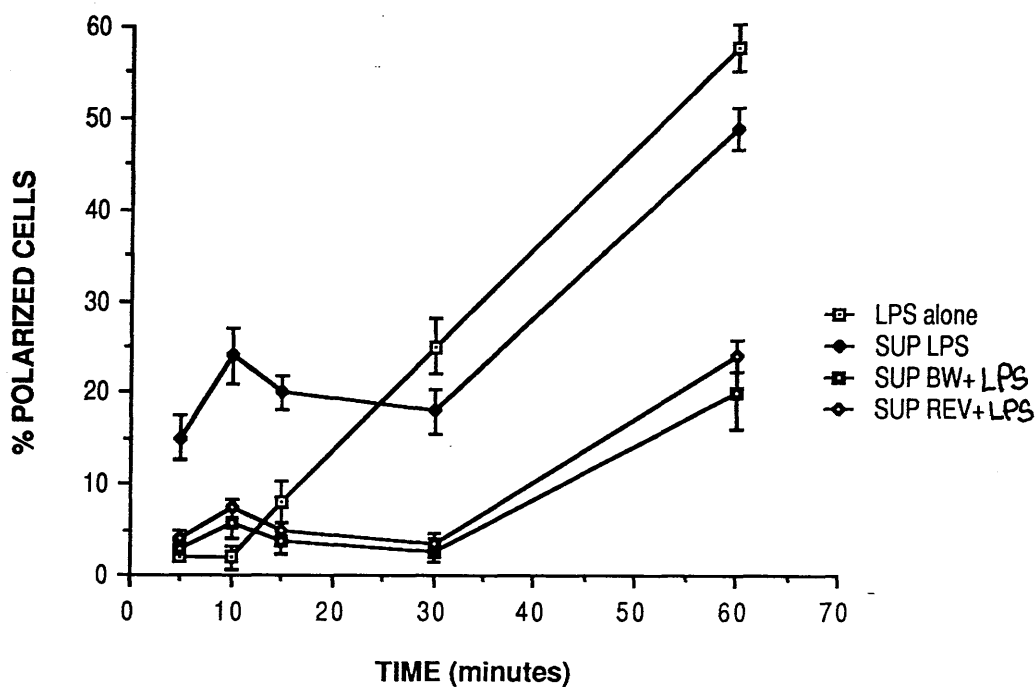


Figure 17. Polarising factor production in the presence of non-steroidal anti-inflammatory drugs represented by the polarisation assay of the supernatant obtained from preincubating PMN (10^6 cells/ml) with 10 ug/ml of drugs for 90 minutes at 37° C. New batch of cells at 10^6 cells/ml was used in the polarisation assay. (n= 4)

Section 7

PRELIMINARY CHARACTERISATION OF THE CELL-RELEASED FACTOR

Further studies of the cell-released factor in the supernatant was needed to characterize the polarising inducer of the neutrophil that may be vital in the inflammatory processes.

Heat treatment

The supernatant saved was heated at 56° C for 30 minutes and 100° C for 3 minutes after which it was tested for the presence of the polarising inducer on the cells. Under both conditions the polarising activity of the PMN was unaffected suggesting that the cell-released factor was heat stable.

Dialysis of cell-released factor

Through dialysis, a rough estimate of the molecular size of the factor could be obtained. The supernatant was dialysed against HBSS/MOPS+HSA for 48 hours changing the medium every 24 hours. After dialysis, the dialysate was incubated with fresh PMN for short-term polarisation assay. Polarisation of the cells was noted in the dialysate while the medium outside the dialysing tubing failed to induce any shape change to the leukocytes. From this observation the factor might be a large molecule, not small enough to diffuse out of the

dialysing bag or a small molecule firmly bound to a large molecule thus preventing dialysis.

Hexane extraction

Equal volumes of the supernatant and hexane were mixed in a separating funnel to allow any organic compound in the supernatant to be isolated from the aqueous portion of the supernatant. The aqueous layer was eluted out and mixed with another volume of hexane before eventually saving it to be used in the polarisation assay. After separation from the hexane layer and concentrating the aqueous layer, a time course of polarisation assay was done using the latter. Minimal activity was detectable ($12.33\% \pm 2.05$) after 60 minutes of incubation with the hexane/buffer system as the control giving the usual background polarising activity of around 2% while the unextracted supernatant recorded $37.33\% \pm 2.5$ polarisation at the same time. It was therefore possible that the factor may not be composed of just a single molecular entity but a mixture or combination of more than one compounds, one of which separates into the aqueous layer and one into the hexane layer. Alternatively, activity may have been destroyed during the extraction.

Thin layer chromatography (TLC)

The organic layer separated after hexane extraction was pooled together, concentrated under pressure in a rotary evaporator and eventually spotted on a TLC plate. Known samples of LPS, lipid A and LTB₄ were also spotted on similar TLC plate as controls. Migrated spots of the tested material were observed under ultraviolet light before spraying the plate with a chemical detector and heating in an oven. Two black charred migrated spots were apparent from the spotted point of the organic layer but none was observed from the controls (Figure 18). The extraction procedure thus suggested that the factor was different from the known control but the technique did not give detailed information about its nature.

Sephadex G-25 gel filtration

To obtain preliminary information about the molecular weight of the active material, gel filtration was performed using Sephadex G-25 to separate large (> 5000 molecular weight) from small molecules. The eluted fluid was first analysed for protein content by a spectrophotometric method at 280 nm and later used in the polarisation assay. As depicted in figure 19, high molecular weight compound having the polarising activity was isolated from the eluant. However, polarisation of the cells was also detectable in the low molecular

weight region signifying a heterogeneity in the polarising activity with different activity at different molecular weight levels. Using the linear relationship between K_{av} and log molecular weight (Determann, 1968), the calculated molecular weight of the small molecule is approximately 1200. K_{av} represents the fraction of the stationary gel volume which is available for diffusion of a given solute species: $K_{av} = (V_e - V_o) / (V_t - V_o)$. V_e is the elution volume of a given solute, V_t is the total volume of the packed column bed and V_o is the void volume which is the elution volume of molecules only distributed in the mobile phase because of their large sizes.

Discussion

Neutrophils in the presence of LPS seem to release a factor or factors that are capable of inducing shape change. This cell-released factor was further studied to understand its nature. Heating the supernatant at two different temperatures did not abolish the polarising capacity of the factor implying that the active component of the factor is heat stable. By dialysing the supernatant, the active component of the factor was retained within the tubing indicating that the factor may be a large size molecule or a small molecule bound to a macromolecule.

Hexane extraction was done as a preparative stage in separating the active component of the released factor using TLC. The aqueous layer separated by the technique was found to have a diminished PMN polarising activity. It is therefore likely that most of the active compound was extracted out of the supernatant and remained in the organic layer. Concentrating this organic portion by rotary evaporator allowed an adequate sample application on TLC plate. Spots of the migrated compound detectable by spraying with concentrated acid revealed the presence of an unknown material different from the known controls of LPS, LTB₄ and lipid A based on the upward migration of the materials on the TLC plate. Such an isolation procedure nevertheless restricted the use of the separated product in a polarisation assay since the product had been chemically modified after being sprayed and heated in the hot oven. Gel filtration provided the means of separating and purifying any mixed compounds to be used in polarisation assay. Separation took place according to the molecular size; large molecules that were unable to be retained by the gel beads were eluted first from the bed while smaller molecules were retarded since they were able to penetrate into the beads. Two different bands of molecular sizes were found again supporting the view of at least a low molecular weight compound is involved in the shape change activity. Small fraction, which is

hydrophobic on phase separation, has almost no absorbance at 280 nm and is quite likely to be a lipid, especially as lipoxygenase inhibitors reduce its formation. The activity detectable at high molecular weight elution is suggestive of the presence of a protein-like compound released by the cells, but unlike a protein it is not destroyed at 100° C. However, by looking at the physical properties studied the two different compounds may be bound to one another thus not portraying any kind of individual molecular characteristics. Results from the experiments conducted so far present the view that LPS may not just modulate the biological functions of the neutrophils in inflammation but also may involve the recruitment of these inflammatory cells by causing the cells to release a factor that would attract other neutrophils to the site of inflammation.

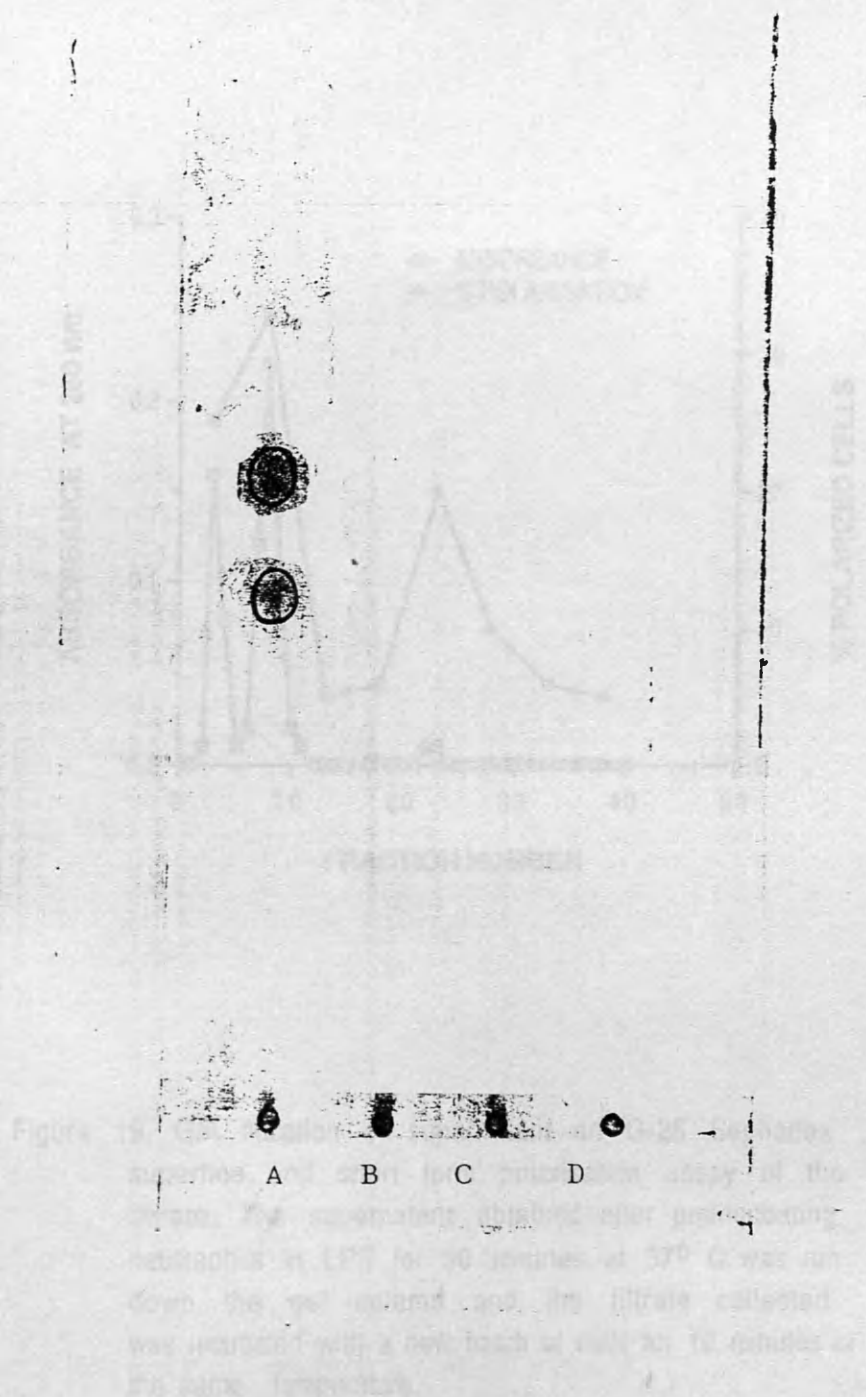


Figure 18. Thin layer chromatography. Silica plate shows the migrated spot (A) of the material extracted from the supernatant by hexane extraction. The spots were revealed by spraying acid and then heated in a hot air oven. Absence of migrated spots from (B) LPS, (C) Lipid A and (D) LTB_4 .

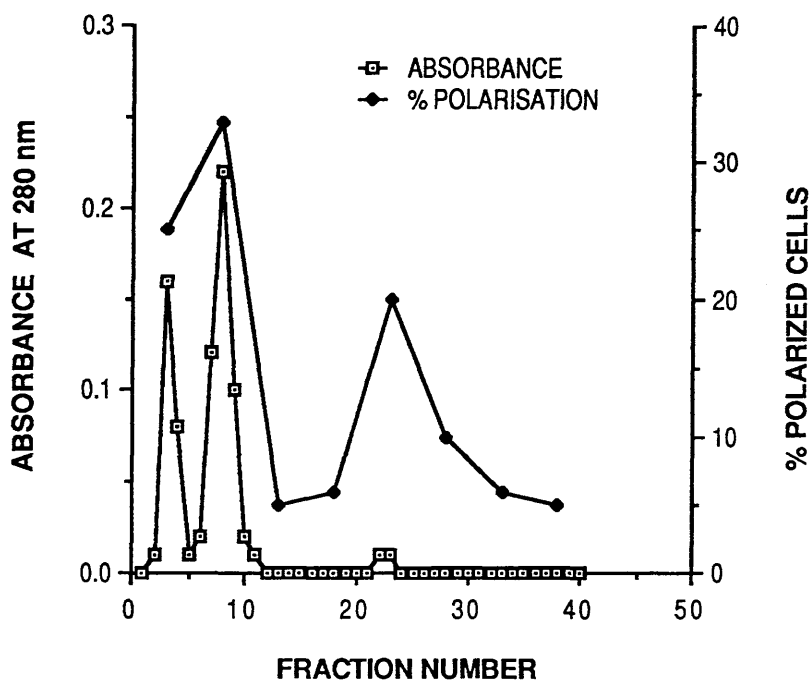


Figure 19. Gel filtration of supernatant on G-25 Sephadex superfine and short term polarisation assay of the filtrate. The supernatant obtained after preincubating neutrophils in LPS for 90 minutes at 37° C was run down the gel column and the filtrate collected was incubated with a new batch of cells for 10 minutes at the same temperature.

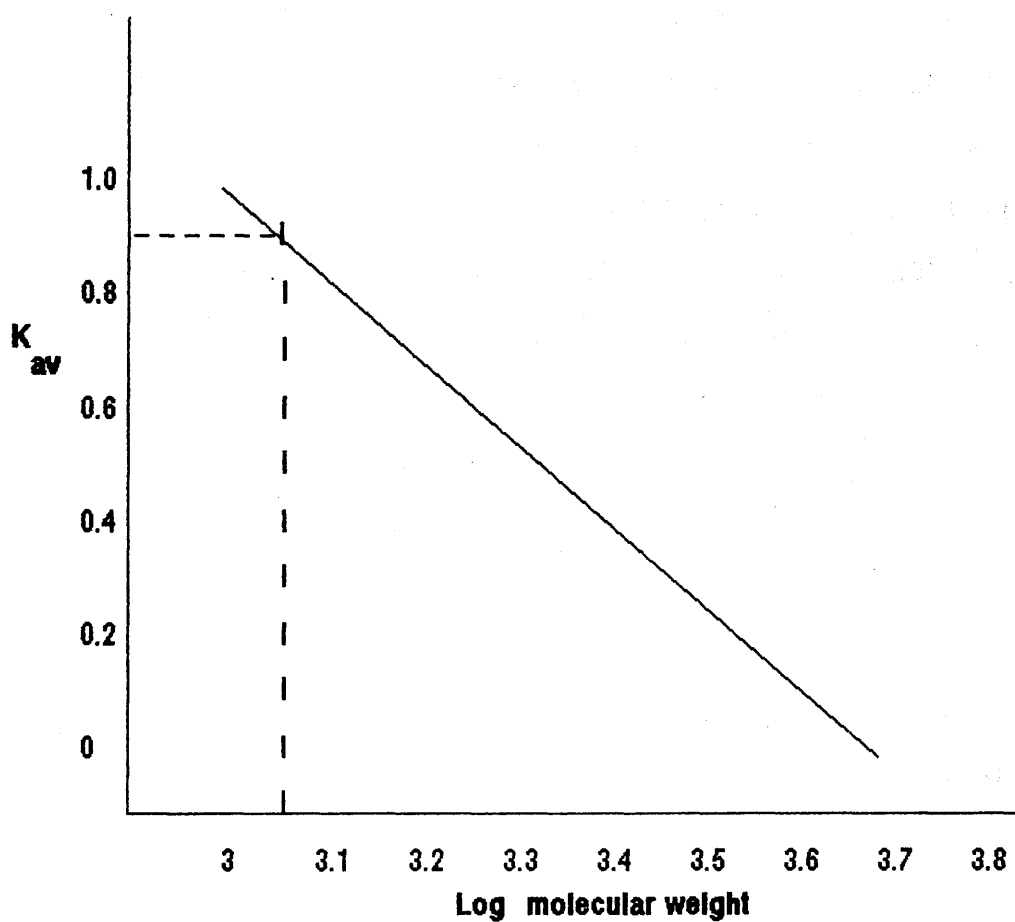


Figure 20. Graph of K_{av} against log of molecular weight.
Using the values from gel filtration assay (mentioned in text)
approximate molecular weight of the compound was determined
based on the linear relationship of log molecular weight and
constant K_{av}

Section 8

RECEPTOR STUDIES

Human phagocytes express a family of cell surface glycoproteins known as LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), and p150,95 (CD11c/CD18). It has been documented that these receptors do bind LPS (Wright and Jong, 1986). Neutrophils from patients lacking in the CD18 family of receptors exhibited the inability to bind to bacterial LPS (Wright et al, 1989). They also discovered that CR3 has a separate binding site that recognizes LPS. It is of interest therefore to test if these receptors do participate in the response of the cells to change shape when encountered with LPS.

Dose response

Cells were preincubated with varying concentrations of CD18 antibodies for 10 minutes at 37° C after which 10 µg/ml of LPS was added prolonging the incubation period for another 60 minutes. Upregulation of the receptors was reported to be maximal after approximately 10 minutes at 37° C (Springer and Anderson, 1986). In figure 21, representatives of the three families of antibodies showed different capacities of inhibiting LPS-induced polarisation of PMN. The inhibitory effect increased as the amount of antibodies increased reaching a maximum inhibition at 5 µg/ml concentration: 33%- CR3

(2LPM19C), 23%- LFA-1 (717), 22%- p150,95 (3.9). Above this concentration the effects were maintained at similar level. Monoclonal antibodies against CD11b/CD18 however presented a better inhibitory effect than that of the other two members of the family. Even though the inhibition was relatively small, at a concentration of 0.1 $\mu\text{g/ml}$ these antibodies showed detectable inhibition of polarisation of the neutrophils.

Time course

For a time course assay, 10 $\mu\text{g/ml}$ of each monoclonal antibody against the CD18 family of receptors was pre-incubated with PMN for 10 minutes and then the PMN were incubated with LPS for the prescribed time period. After 15 minutes of LPS incubation, all three antibodies gave an inhibitory effect of $\geq 70\%$ (Figure 22). As time of incubation with LPS was extended, less inhibition was seen particularly after 90 minutes when anti-LFA-1 anti-p150,95 showed no inhibition but anti-CR3 still possessed slight inhibitory activity.

Specificity of the monoclonals

To ensure that the antibodies were acting specifically towards release of LPS-induced factor by the cells, PMN were challenged first with antibody, then with an irrelevant chemotactic factor, FMLP at 10^{-8} M, whose receptors were unrelated to the CD11/CD18 family .

Polarisation due to FMLP was unaffected after 30 minutes of incubation in the presence of the antibodies (90%-control; 90%- anti-CR3; 92%- anti-LFA-1; 89%- anti-p150,95). Another possible reason for the inhibition was that the inhibitory effect of these monoclonals on LPS-induced polarisation was due to low affinity binding of excess antibodies to irrelevant (non-CD11/CD18) cell surface structures. This was tested by washing the cells after the first preincubation stage (10 minutes at 37° C) to remove any unbound antibodies before eventually continuing the polarization assay using LPS. The results were not altered by this manoeuvre giving a clear signal of the specificity those antibodies acted upon.

Assessment of different monoclonal antibodies

Different batches of antibodies with similar specificities do not always exhibit the same kind of actions on a particular system. Likewise in this project different batches of the antibodies were assessed in their blocking capacities towards LPS-induced PMN polarisation. As would be expected from tables 5a, 5b and 5c antibodies against the CD11b/CD18 receptors displayed inhibitory effects that ranged from 0% to 40% while the range for anti-CD11a/CD18 was from 0% to 30%. Only one particular batch of anti-CD11c/CD18 was able to reduce the polarising activity by 20% whereas most of the monoclonals from this group had no effect at all.

The involvement of CD11a/CD18 receptors in the LPS-induced polarisation is somewhat greater than the other two members of the family.

Effect on the factor production

Reduction in the percentage of polarised cells, presumably by blocking the LPS receptor, would mean that the release of the LPS-induced factor was consequently affected. Testing the supernatant, from cells preincubated with antibodies, then LPS (90 minutes) in short-term polarisation assays on fresh cells showed a significant reduction of the percentage of polarised cells by 37% for anti-CR3, 14% for anti-p150,95 and 20% for anti-LFA-1 (Figure 23). Serial dilutions of the supernatant resulted in reduced activity. Some cell-released factor was therefore released despite the blockage by these antibodies at the earlier stage of exposing PMN to LPS.

Blocking with three antibodies together

A combination of antibodies against all three proteins together would be expected to produce a greater inhibition than each one tested separately. The bar graph of figure 24 indicates that even after 90 minutes of incubation time, the combined antibodies had a higher inhibitory action (up to 34%) than the antibody free control.

Blocking with RGD peptides

Since a member of the CD11/CD18 family of receptors has been found to recognize polypeptide ligands bearing the amino acid sequence Arg-Gly-Asp, the triplet RGD-sequence (Wright et al, 1987), it would be of interest to observe if peptides containing the RGD-sequence would exhibit similar activity to that of the antibodies in inhibiting the polarisation of neutrophils. Four different peptides were chosen to study their effects on cell polarisation namely: Gly-Arg-Gly-Asp-Ser (RGD1), Gly-Arg-Gly-Asp-Pro-Lys (RGD2, a fibronectin analogue), Arg-Gly-Asp (RGD3) and Arg-Gly-Asp-Ser (RGD4). These peptides were used in a similar assay to that for assaying the monoclonal antibodies. Pentapeptide containing cell attachment domains of fibronectin (RGD1) and fibronectin analogue (RGD2) specifically displayed a significantly high inhibitory effect on LPS-induced polarisation of PMN, 40% and 30% respectively, as depicted in figure 25. Interestingly peptides accommodating just the RGD sequence (RGD3 and RGD4) were relatively ineffective in suppressing the activities observed compared to the pentapeptide and the fibronectin analogue proving that the presence of the triplet amino acid sequence may not solely be responsible for the blocking of morphological changes in the cells.

Discussion

Recently members of the CD11/CD18 complex of leukocyte receptors, formerly known as CR3 family receptors, have become of considerable interest in inflammation research. The receptors were shown to be important in mediating adhesion of PMN to endothelial cell lining during inflammation and also found to function in the recognition of several other particles besides the surface-bound complement C3bi (Ross et al, 1985a; Wright and Jong, 1986; Bullock and Wright, 1987; Dana et al, 1986). Recently Wright et al (1989) observed that the CD11/CD18 receptors expressed the binding site for LPS which is distinctly different from the binding site for C3bi. Among the three members of the CD11/CD18 glycoproteins used in the assays, antibodies against CD11b/CD18 displayed greater effect in inhibiting the polarisation activity. This was in accordance with the findings that CD11b/CD18 functional abilities are mainly associated with PMN cellular functions while CD11a/CD18 and CD11c/CD18 are generally linked to cytotoxic effects of other inflammatory cells (Wright and Detmers, 1988). Other monoclonal antibodies within the same family group showed variable effects in blocking LPS-induced polarisation of neutrophils. Eight out of twelve of the CD11b/CD18 antibodies tested were capable of modulating the response; 4 out of 7 of the CD11a/CD18 group were

able to cause any significant blocking while only 1 out of 8 of the CD11c group was able to show significant blocking capacity.

A decline in the inhibitory effect for the antibodies at the later stage of incubation could be attributed to the aspect of interaction of antibodies since it may be that binding of the antibodies to cell surface molecules is reversible. Thus instead of completely blocking a molecule's function, the antibodies may hinder the effect. Antibodies could also be endocytosed by the cells at this stage. Difference in the degree of inhibition between the 3 monoclonal antibodies could be that the interaction by the anti-CR3 to PMN is much more efficient than the other 2 members of the group. The specific action of the antibodies towards the receptors that are required for LPS to interact with on the target cells need to be assessed so as to exclude the non-specific actions of the antibodies. Such antibodies specificities were obvious when FMLP, a known chemoattractant with specific receptors (Koo et al, 1982), was introduced to observe any blocking effect by the antibodies in which the cell responses directed towards FMLP was left undisturbed. Excessive amount of unbound antibodies which may have contributed to a non-specific blocking effect towards cellular polarisation was shown not to have been involved. This assessment was based on the polarization

assay conducted after the antibodies which had been preincubated earlier with the cells to block the antigens were washed off. Both aforementioned specificity tests affirmed the exact binding target of these antibodies towards the receptor sites required for the recognition of LPS molecules. Effective binding of the antibodies towards cell surface receptors, probably blocking access of LPS interacting, would thereby down modulate the production of the released factor. Reduced percentage of polarized PMN noted in the polarization assay on the supernatant was an evidence of such interference. Nevertheless, blocking actions brought about by the antibodies were incomplete since polarization of PMN was still present when the supernatant was serially diluted. Interaction of LPS with the cell membrane of PMN looks to be importantly required for the release of the shape change inducer.

Three monoclonal antibodies together suppressed shape change more efficiently than individual antibodies. Here all the three sites seemed to be blocked simultaneously thus reducing the probability of LPS to interact with the receptor sites. Similar observations were reported by Wright and Jong (1986) in macrophages in which profound inhibition of LPS binding was only seen when all three family members were involved. Data published also indicate that CR3 recognizes a RGD-containing sequence molecule (Wright et

al, 1987). Addition of RGD-containing peptides to the assay system would be expected to affect the polarising activity. As shown in figure 25, strong inhibitory actions were presented by those peptides particularly associated with fibronectin. New evidence indicates high structural homology between the β subunits of the CD18 complex with adhesion-promoting proteins such as fibronectin receptors that recognize the RGD sequence (Kishimoto, 1987b; Law et al, 1987). Hence, the information obtained supports the role of recognizing an RGD sequence in the LPS-induced PMN polarization. Separate binding sites for two different ligands within a single receptor but functionally linked could account for such reaction as observed by Wright et al (1989). Another possibility that could explain the contrasting observation may be linked to a newly discovered receptor that has ligand binding specifically for the RGD sequence expressed by human neutrophils (Gresham et al, 1989). This signal-transducing molecule could be interrelated with the activation of PMN induced by the presence of LPS. Cumulative results implicate the role of CD11/CD18 family of receptors on PMN in recognising bacterial LPS that would consequently induce traditional locomotive morphology for the leukocytes.

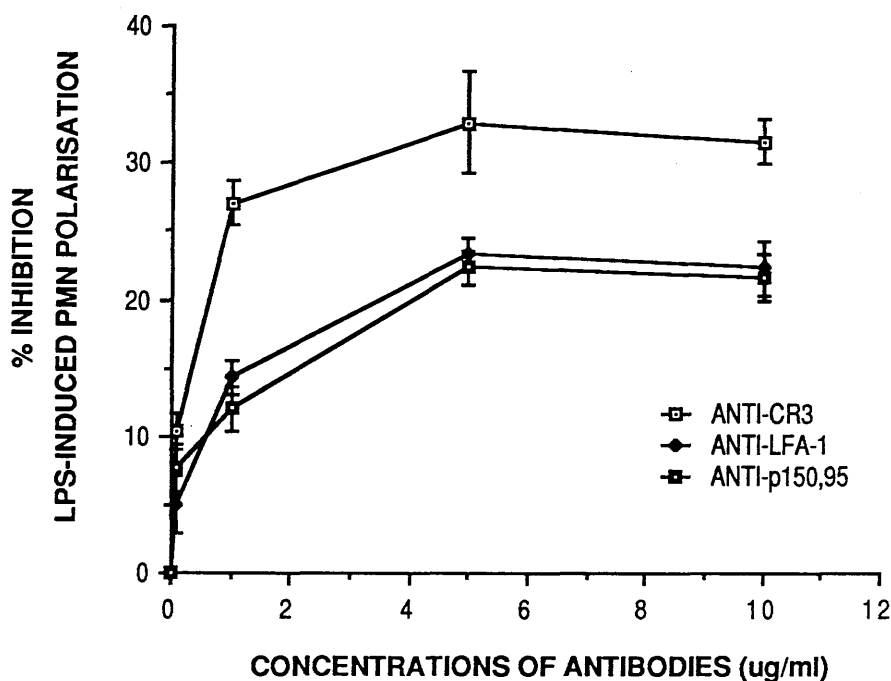


Figure 21. Dose response curve of monoclonal antibodies in the blocking activity of LPS-induced polarisation assay. The antibodies against CD11 / CD18 glycoproteins were added to suspensions of neutrophils (10^6 cells/ml) and incubated for 10 minutes at 37° C before finally adding 10 ug/ml LPS continuing the incubation period for another 60 minutes. The experiments were done in triplicate. Bars represent standard deviation values. Antibodies codes : 2LPM19C (anti-CR3), 717 (anti-LFA-1), 3.9 (anti-p150,95)

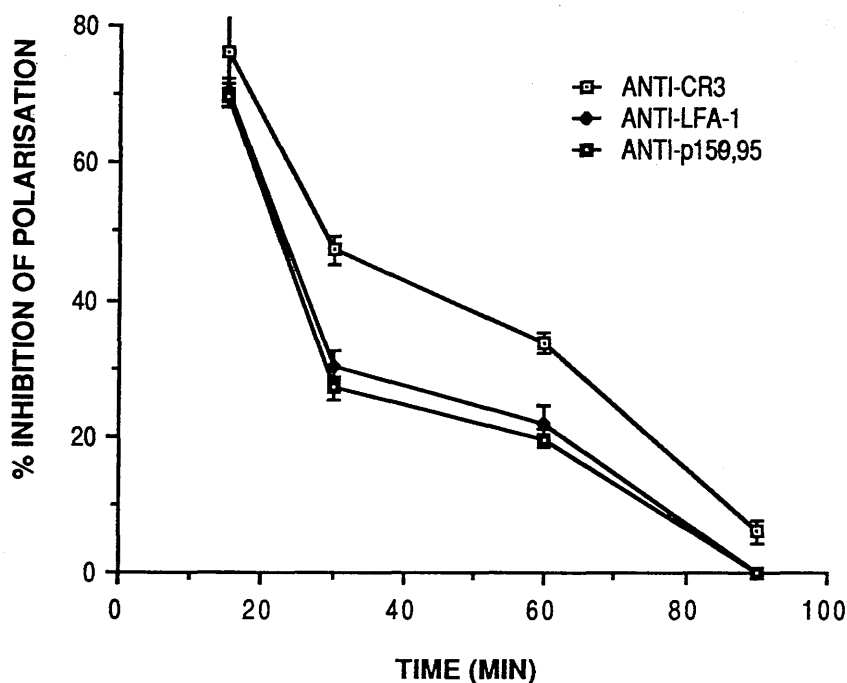


Figure 22. Time course in the blocking activity of LPS-induced neutrophil polarisation by the CD 11/CD 18 monoclonal antibodies. Monoclonal antibodies (10 ug/ml) were incubated with PMN for 10 minutes at 37° prior to adding LPS (10 ug/ml). The experiments were done in triplicate. Antibodies codes: 2LPM19C (anti-CR3), 717 (anti-LFA-1), 3.9 (anti-p150,95)

Antibody code	Specificity	Percent inhibition of LPS-induced neutrophil polarisation	Types of chains
648 (44)	CD11b	38.6	α
649 (VIM12)	CD11b	28.8	α
650 (52.94)	CD11?	30.0	
651 (TM15)	CD11b	37.1	α
652 (KiM5)	CD11b	None	α
653 (M522)	CD11b	37.1	α
654 (LM2/1.6.11)	CD11b	17.2	α
655 (M3D11)	CD11b	None	
656 (MO1)	CD11b	None	α
657 (B2.12)	CD11b	None	α
2LPM19C	CD11b	39.7	α
Mo1	CD11b	18.4	

Table 5a. Assessment of blocking activity of individual monoclonal antibodies against CR3 on neutrophil polarisation. The antibodies (10 μ g/ml) were preincubated with neutrophils (10⁶ cells/ml) for 10 minutes and then mixed with 10 μ g/ml LPS for 60 minutes at 37° C.

Antibody code	Specificity	Percent inhibition of LPS-induced neutrophil polarisation	Types of chains
706 (60.3)	CD18	None	β
710 (CLB.54)	CD18	None	β
713 (M232)	CD18	None	β
719 (TS1)	CD18	None	β
725 (IB4)	CD18	None	β
711 (CIMT)	CD11a	5.1	α
714 (LeoLFA-1)	CD11a?	None	
715 (25.3.1)	CD11a	2.4	α
717 (2F12)	CD11a	28.9	α
720 (22.1.1.17)	CD11a	19.4	α
723 (6.1.2)	CD11a	None	α
724 (CLB-LFA-1)	CD11a	None	α

Table 5b. Assessment of blocking activity of individual monoclonal antibodies against the common beta chain (CD18) and LFA-1 (CD11a) of the adhesion promoting receptors on neutrophil polarisation following similar procedures to that of anti-CR3 antibodies.

Antibody code	Specificity	Percent inhibition of LPS-induced neutrophil polarisation	Types of chains
726 (3.9)	CD11c	21.4	α
256 (BU15)	CD11c	None	
277 (KB23)	CD11c	None	
300 (Ki-M1)	CD11c	None	
721 (SHCL-3)	CD11c	None	α
727 (L29)	CD11c	None	α
728 (3KB43)	CD11c	None	α
729 (KB23)	CD11c	None	α
730 (KB90)	CD11c	None	α

Table 5c. Assessment of blocking activity of individual monoclonal antibodies against p150,95 (CD11c) on neutrophil polarisation. Similar methods were used as that of the previous antibodies.

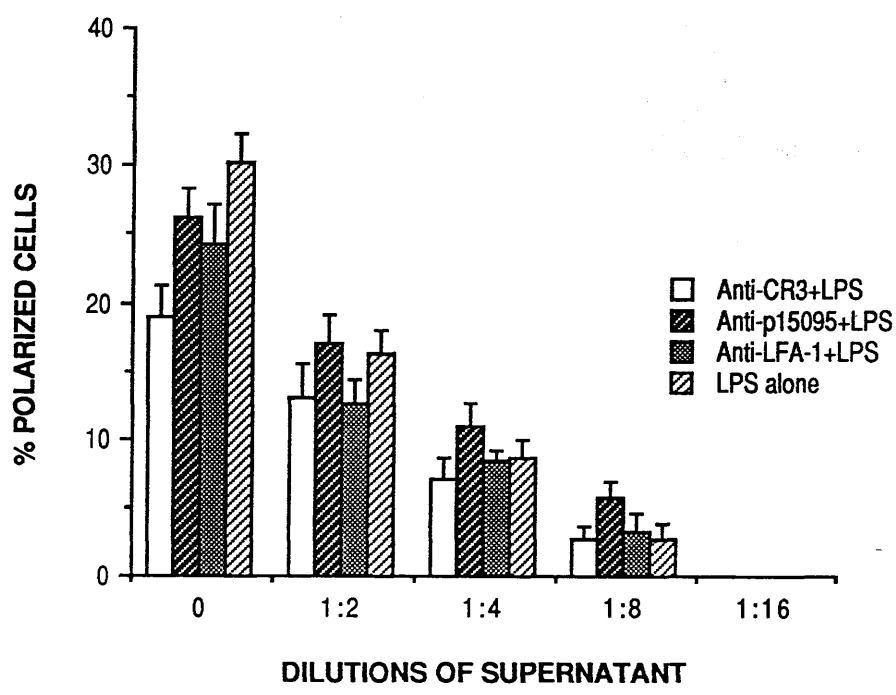


Figure 23. Effect of diluting supernatant in the presence of monoclonal antibodies on neutrophil polarisation assay. The supernatant was obtained by preincubating PMN (10^6 cells/ml) in 10 ug/ml of antibodies for 10 minutes at 37° C and then adding 10 ug/ml LPS continuing incubation for 90 minutes at the same temperature. This supernatant was then incubated with a new batch of PMN (10^6 cells/ml) for 10 minutes at 37° C. The values depicted were from 3 different experiments. Bar are the standard deviations of the means. Antibodies codes: 2LPM19C (anti-CR3) , 717 (anti-LFA-1), 3.9 (anti-p150,95)

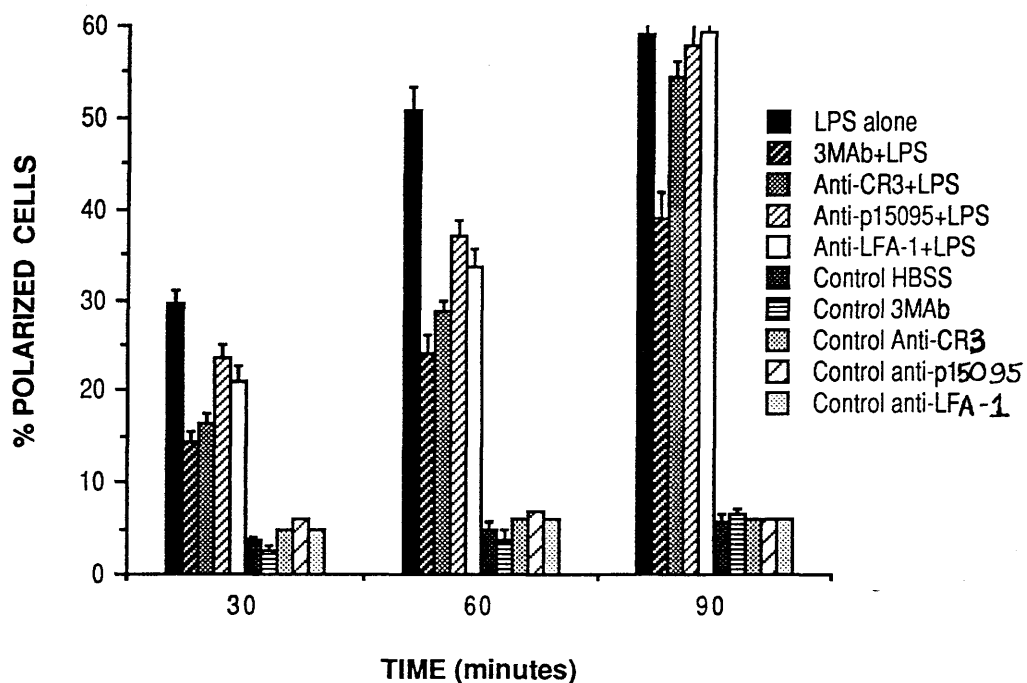


Figure 24. Blocking activity on LPS-induced neutrophil polarisation of three monoclonal antibodies together in comparison to each individual blocking capacity. Three antibodies combining together (10 ug/ml each) were preincubated with PMN for 10 minutes at 37° C before exposing the cells to LPS (10 ug/ml). Individual antibodies was treated similarly to that of the combined antibodies (n=3). Bars represent standard deviation values. Antibodies codes: 2LPM19C (anti-CR3), 717 (anti-LFA-1), 3.9 (anti-p150,95)

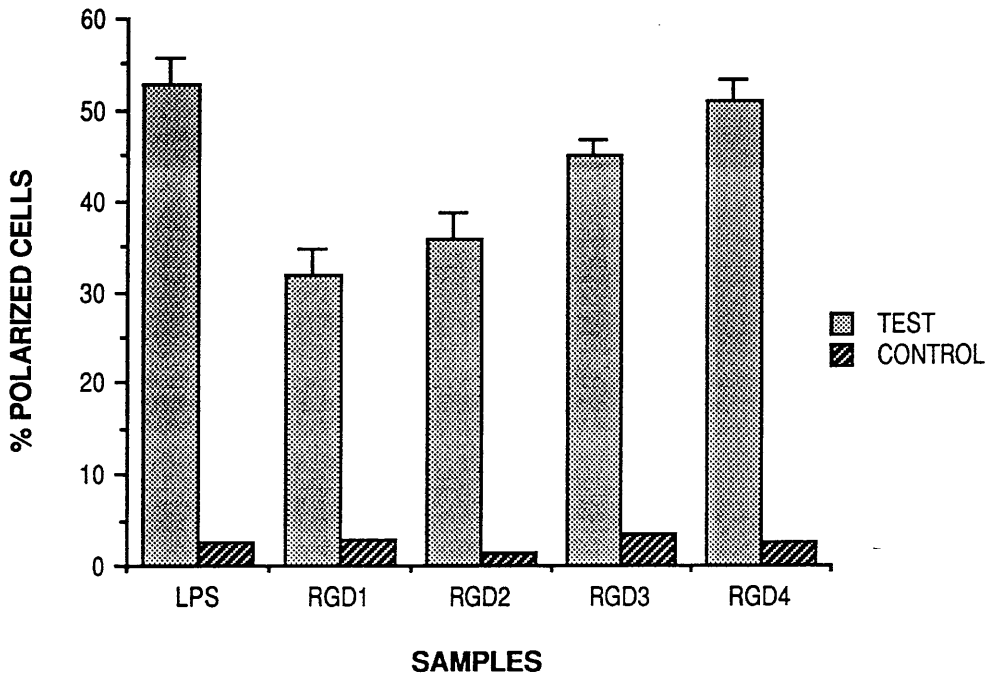


Figure 25. Blocking assay of LPS-induced PMN polarisation using RGD-containing peptides. The cells (10^6 cells/ml) were exposed to the peptides (10 ug/ml) for 10 minutes at 37° C followed by the addition of LPS (10 ug/ml). The mixture was further incubated for 60 minutes at similar temperature. Results from five different experiments were used to plot the chart along with the standard deviation values shown by the bars. RGD1= Gly-Arg-Gly-Asp-Ser; RGD2= Gly-Arg-Gly-Asp-Pro-Lys (fibronectin analogue); RGD3= Arg-Gly-Asp; RGD4= Arg-Gly-Asp-Ser.

Section 9

POLARISATION ASSAY ON DIABETIC CELLS

Thus far the project has shown that LPS-induced polarisation of PMN may be inhibited by blocking the cell-surface receptor sites for the LPS molecules. The methodology applied could be considered as simulating a defective neutrophil function. Hence, the assay system was tested on cells which are known to be defective in vivo. Diabetics PMN are believed to be among those that have a depressed functional capacity as cited in the introductory part of the thesis. In a study involving diabetic patients, three biochemical indicators are frequently included in analysing the experimental results: levels of blood glucose, glycosylated haemoglobin and fructosamine. Raised blood glucose concentration above levels (normal range 3.2 - 5.3 mmol/lit) proposed by the Expert Committee of the World Health Organization forms an indicator in diagnosing diabetes mellitus (Report of WHO, 1985). Levels of glycosylated haemoglobin (normal range 5.7% - 8.2%) has been shown to reflect the degree of hyperglycaemia in diabetic patients thus presenting a satisfactory indicator of the metabolic control of the patients (Gonen et al, 1977; Graf et al, 1978; Paisey et al, 1980). The fructosamine assay described by Johnson et al (1982) provides an index of glycaemia capable of

distinguishing normal (range 1.1 - 1.6 mmol/lit) and diabetic populations. Both elevated levels of fructosamine and glycosylated haemoglobin in diabetics were found to be highly correlated in a positive manner by the above mentioned investigators with the blood glucose concentrations.

When PMN from diabetic patients of type 1 (insulin-dependent) and type 2 (non-insulin dependent) were incubated with 10 μ g/ml LPS for 60 minutes, fewer cells polarized than in the normal non-diabetic individuals (Table 6a and Table 6b). On the average, the IDDM patients had higher percent reduction of polarisation than the NIDDM patients (mean values 41% and 28.6% respectively). Three main clinical parameters, glycosylated haemoglobin (HbA1), fructosamine and blood glucose levels, were considered in the study to observe if their levels were related to the reduction in the proportion of polarised cells. Levels of HbA1 and fructosamine in NIDDM and IDDM did not appear to be positively correlated with the reductive effect of LPS-induced PMN polarization (Figures 26 and 27; statistically non-significant ($P > 0.05$)). In vitro assay of the effect of glucose concentration on LPS-induced PMN polarizations was conducted since blood glucose level has been implicated as a contributor to the derangement of PMN functional capacities (Kjersem et

al, 1988). Normal neutrophils were initially exposed to glucose at different concentrations for 10 minutes at 37° C before being challenged with either 10 µg/ml LPS for a period of 60 minutes or 10^{-8} M FMLP for 30 minutes. As depicted in figure 28, at 3 mg/ml (16.7 mmol/lit) of glucose there was a significant reduction of 23% in cell polarization with this inhibitive effect raised up to 87% at 30 mg/ml. Unlike the result from LPS, only at 20 mg/ml (110 mmol/lit) of glucose was there a significant 19% reduction of FMLP-induced polarisation. However, this needs a control for the inhibitory effects of non-glucose-containing hyperosmolar solutions.

PAF modulation

Platelet activating factor (PAF), a pro-inflammatory lipid mediator produced by a variety of cells including platelets, basophils, monocytes and PMN, has been demonstrated to possess a stimulatory effect on human PMN functions such as exocytosis, migration and superoxide production (Shaw et al, 1981). PAF not only directly activates PMN but in small quantities "primes" them as well eventually allowing PMN to respond to subsequent stimuli (Vercelloti et al, 1988). Based on the reported observations of these workers, actions of PAF were therefore investigated in the polarization assays using both the normals and the diabetic cells. Both types of cells were preincubated with 10^{-8} M PAF for 10 minutes before adding LPS. Normal

PMN exhibited an average of 44% increase in the number of polarised cells (Figure 29). Representatives of seven IDDM patient cells showed similar effects but with varying increases in the percent polarisation ranging from an increment of 55% to 103%. Even though PAF on its own does cause shape change, the percentage of these changes were within the control limit of $< 10\%$. Any cells with shape changes of $> 10\%$ at the beginning of the experiments were discarded.

Visual assay

The same method described earlier to measure shape changes of normal cells in suspension was used to study cells from diabetic patients. The illustrations in figure 30 of IDDM cells show no apparent morphological differences from the shapes of the normal PMN (Figure 11). The frequency of shape-change though was much less for the diabetic cells than the normal counterparts.

Discussion

Diabetic neutrophils manifested diminished PMN functional capacities as the disease progresses. Hence, the projection is that diabetics PMN would be responding poorly too in the LPS-induced polarisation assay. The results from tables 6a and 6b clearly demonstrated the functional defects of the diabetic neutrophils to polarize as well as normal cells. Elevated levels of

fructosamine and glycosylated haemoglobin did not appear to contribute to the poor response of LPS-induced PMN polarization as indicated by the correlation tests. Blood glucose levels that have long been thought to have a substantial effects on neutrophil functions in diabetes mellitus patients failed to show any positive correlation with this defect even though experimental data using glucose to interfere with the assay system gave a positive effect. Manoeuvring glucose concentration in vitro may not actually simulate the in vivo situation exactly. The in vitro studies using exogenous glucose simultaneously showed that FMLP has a different target site than LPS since the inhibitory effect was obtained at a much higher concentration using FMLP than using LPS. A similar inhibitory effect had earlier been displayed when monoclonal antibodies against CD11/CD18 receptors were used in the assay. From the study it seems that the receptors needed for LPS binding on PMN may be poorly expressed or poorly functional in the diabetic cells. The reason behind this defect still remains unclear.

Besides having the ability to potentiate neutrophil responses to subsequent activation by an agonist, PAF was taken into consideration as a factor for assay following the work that showed PAF increased expression of membrane receptors for CR3 in human PMN (Shalit et al, 1988; Vercelloti et al, 1988). Moreover, unlike

other biological compounds that do increase expression of this family of receptors, PAF on its own did not cause shape change above that of the control level. Preincubating both the normal or diabetic PMN with PAF before challenging with LPS resulted in the increase of numbers of polarized cells. This marked enhancement of polarization is an indication of a stimulating or priming effect of PAF on neutrophils particularly in the case of diabetic cells. If membrane receptors do in fact play a role in the LPS-induced PMN polarization, then the action of PAF in enhancing the response could be partly due to the increased in cellular expression of CR3 receptors as documented by previous investigators (Shalit et al, 1988; Vercelloti et al, 1988). Another possibility lies in the biochemical aspect of cellular activation in which PAF has been proposed as a priming stimulus via activating protein kinase C (Gay et al, 1986). This phenomenon could have potentiated the polarizing response of the PMN for both normal and diabetic individuals. The locomotive apparatus in diabetic cells did not show any behavioural abnormalities judging from the motion of the patient cells in suspension with the aid of videotaping. Motile

activity though was somewhat less which may indicate a low cellular activating process. The observations thus far reflect poor metabolic control as a result of impaired utilization of glucose to turn on the biochemistry of the cell (Esmann, 1963).

Patient	HbA1	Fructosamine	Blood glucose	Percent reduction of polarisation
P1	10.1	3.6	17.4	20.0
P2	12.1	3.9	18.1	30.0
P3	10.3	4.3	17.2	28.0
P4	10.3	3.3	11.5	28.0
P5	8.1	3.4	12.9	15.4
P6	12.7	4.8	23.2	40.0
P7	12.5	3.4	13.7	20.0
P8	12.7	3.6	6.3	31.7
P9	7.6	2.8	10.3	32.3
P10	6.9	2.6	11.3	23.1
P11	12.0	3.7	11.1	32.3
P12	5.4	2.3	5.1	33.3
P13	11.2	3.7	13.5	28.6
P14	11.5	4.1	15.5	26.7
P15	6.2	3.1	11.5	38.9
P16	13.5	5.0	19.7	27.8
P17	11.2	-	11.8	30

Table 6a. Polarisation assay on non-insulin dependent diabetic cells. Patient cells were incubated with 10 μ g/ml of LPS for 60 minutes along with a control normal cells. Percent polarisation was then converted into percentage of reduction. HbA1 (glycosylated haemoglobin) is expressed in percent, fructosamine and blood glucose in mmol/lit.

Patient	HbA1	Fructosamine	Blood glucose	Percent reduction of polarisation
P1	9.7	3.9	12.3	36.7
P2	8.5	3.2	14.1	35.2
P3	9.8	3.8	23.6	39.0
P4	9.0	3.2	20.3	70.6
P5	8.3	2.9	7.2	64.8
P6	8.7	3.4	15.7	28.8
P7	-	3.9	20.3	42.3
P8	10.4	3.8	3.5	32.1
P9	9.5	-	19.8	37.5
P10	9.8	3.9	22.4	32.1
P11	9.7	4.2	10.0	23.2
P12	8.2	3.1	15.3	48.0
P13	7.3	2.6	11.0	64.0
P14	16.2	4.0	23.0	35.7
P15	9.3	3.5	14.6	25.0

Table 6b. Polarisation assay on insulin dependent diabetic cells. The treatment of these cells was the same as that of non-insulin dependent diabetic cells.

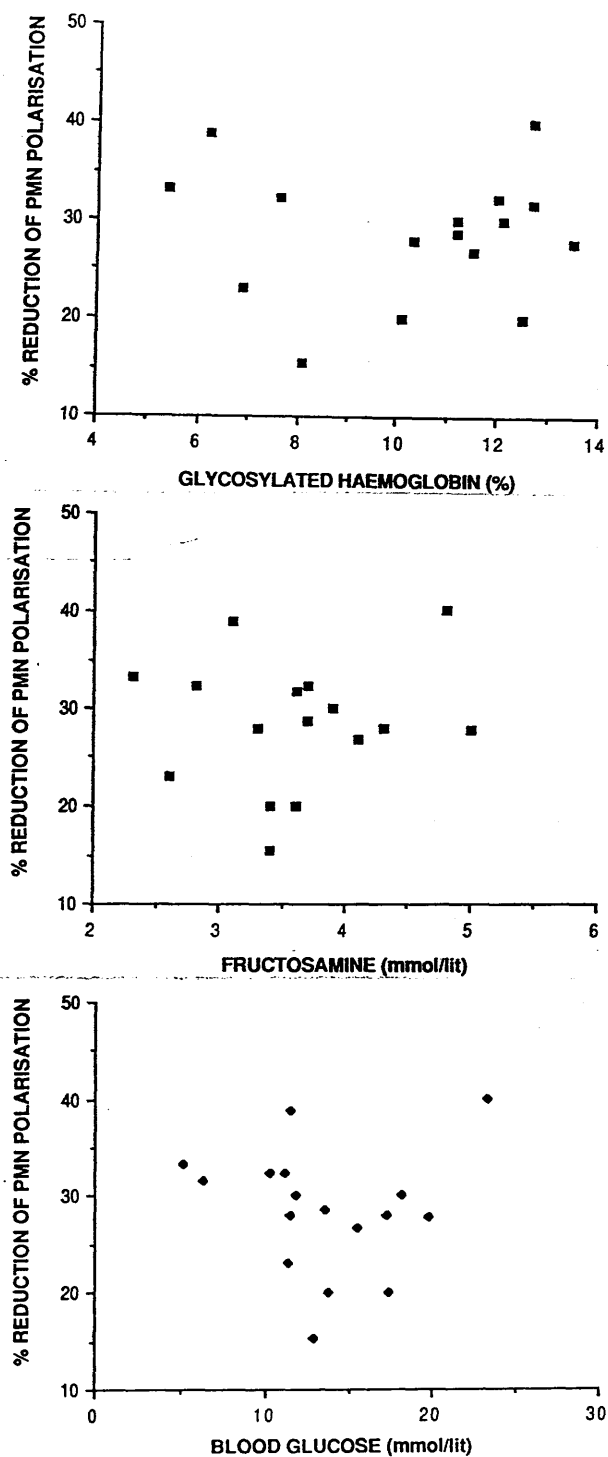


Figure 26. Comparison of the percent reduction in PMN polarisation with the level of glycosylated haemoglobin, fructosamine, and blood glucose of non-insulin dependent diabetics. (Correlation test, not significant. $P > 0.05$)

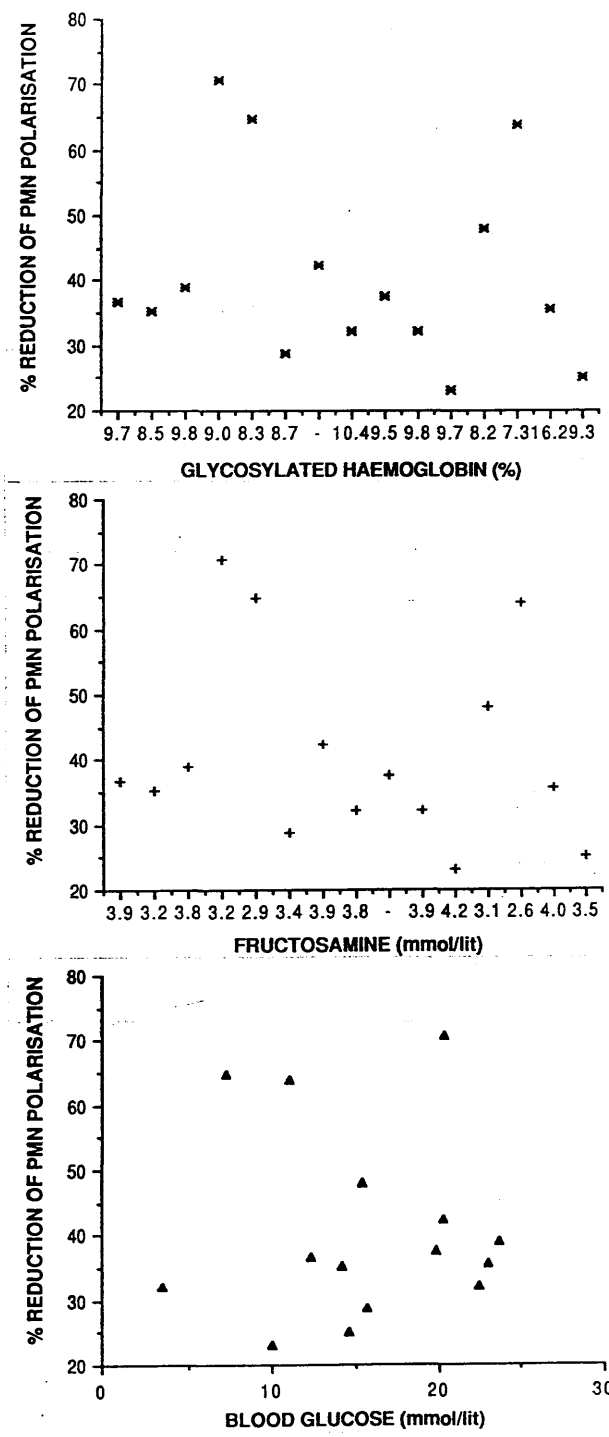


Figure 27. Comparison of the percent reduction of PMN polarisation with the level of glycosylated haemoglobin , fructosamine , and blood glucose of insulin-dependent diabetics. (Correlation test, not significant. P >0.05)

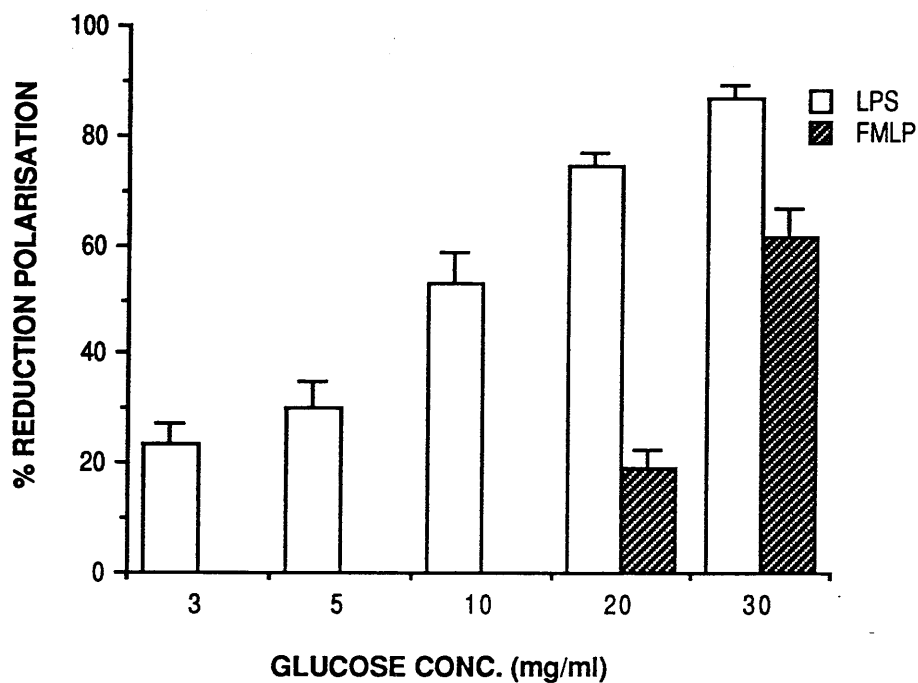


Figure 28. Dose response effect of glucose on LPS-induced and FMLP-induced PMN polarisation. Neutrophils at 10^6 cells/ml were exposed to glucose for 10 minutes at 37°C before being challenged with LPS (10 $\mu\text{g/ml}$) for 60 minutes or FMLP (10^{-8} M) for 30 minutes maintaining the same temperature. Data collected were from 4 different experiments. Standard deviations of the means are depicted by the error bars.

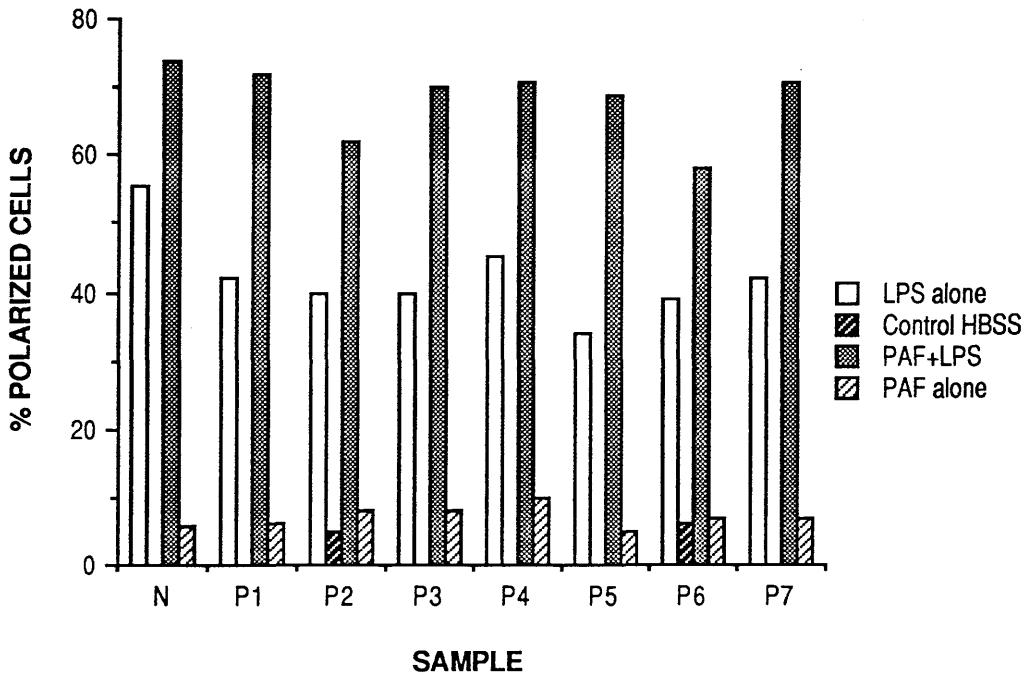


Figure 29. Polarisation assay of neutrophils in the presence of LPS and PAF for normal (N) and patients (P) cells. Cells ($10^6/\text{ml}$) were preincubated with 10^{-8} M PAF for 10 minutes at 37° and then challenged with 10 $\mu\text{g}/\text{ml}$ LPS for 60 minutes at the same temperature.

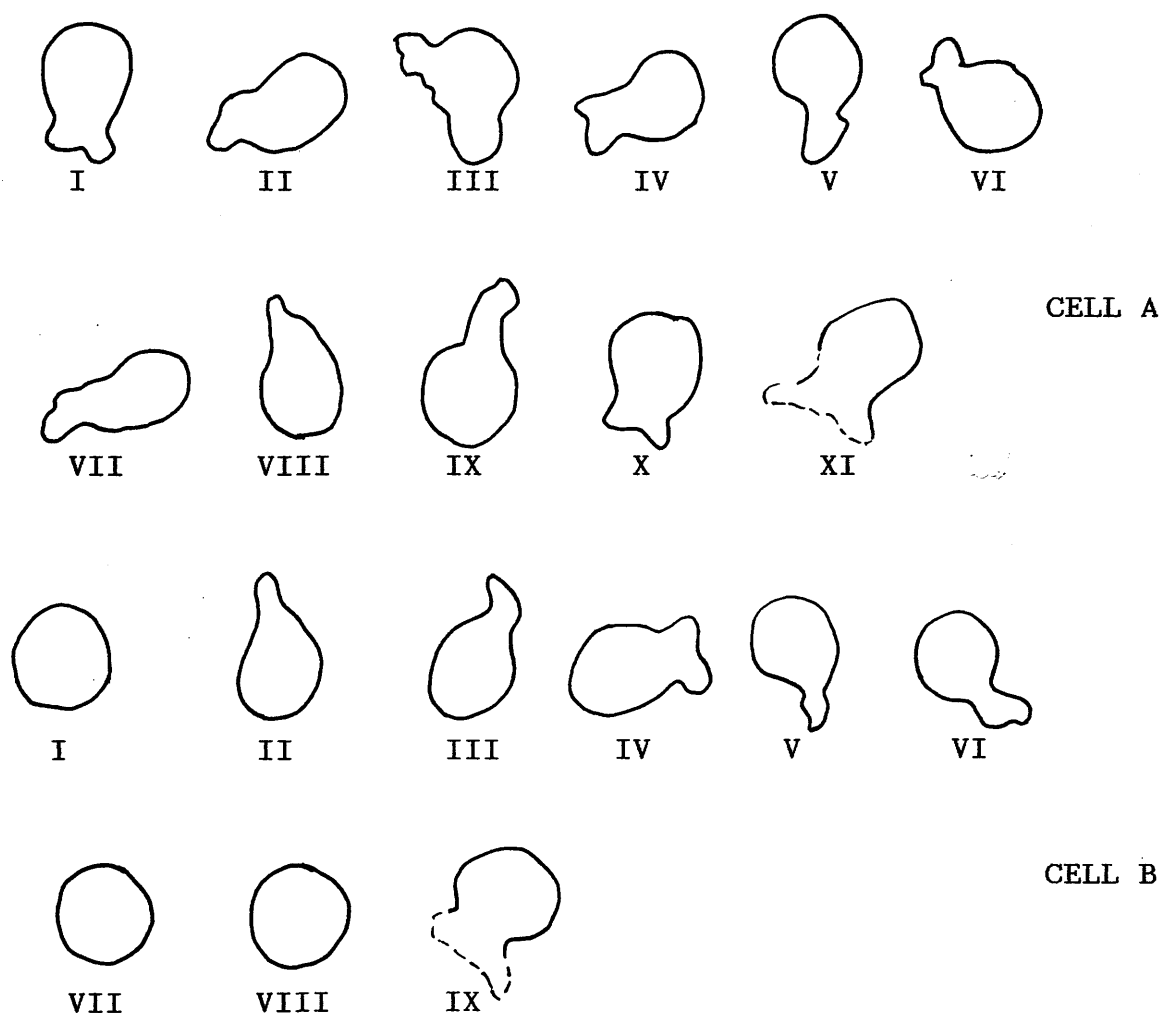
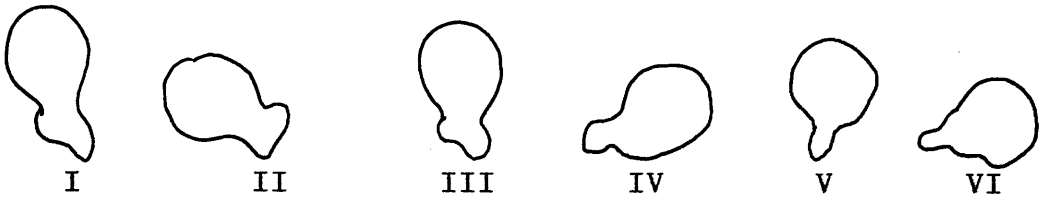
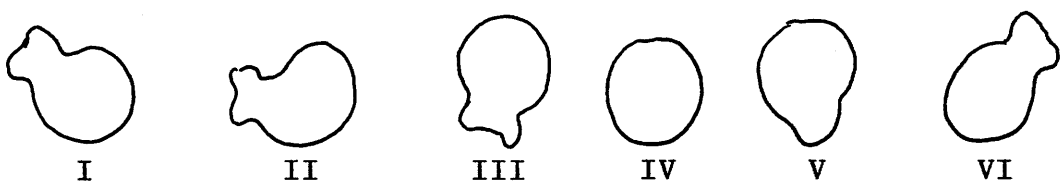
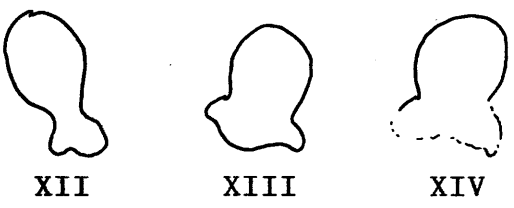
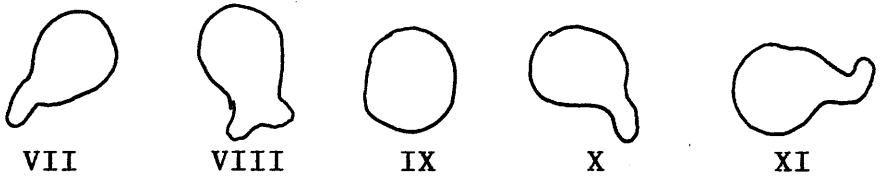


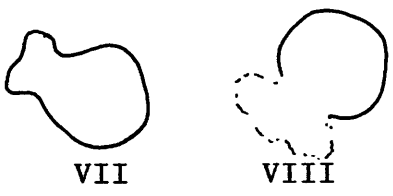
Figure 30. Live diabetic cells using videotape. PMN from IDDM patient were incubated at different times with LPS before observing in filming chamber and later videotaped. Motions of the cells in suspension were traced out at 30 seconds intervals (Roman numerals). Period of incubation: Cell A- 30 min, Cell B- 60 min, Cell C- 90 min and Cell D- 120 min.



CELL C



CELL D



Section 10

CHEMILUMINESCENCE ASSAY

Chemiluminescence is an energy product of phagocyte oxygenation activity. Measurement of this light emission enables monitoring of phagocyte oxygenation activity which may originate on contact with the particles to be ingested. The measurement requires a reasonable number of cells and a sensitive light detector. Chemiluminescence measurement can be increased even further by the addition of luminol which markedly amplifies the luminescence response (Harvath and Andersen, 1979).

Dose and time responses to a particulate stimulus

Zymosan at 4 mg/ml was opsonized with varying serum concentrations before mixing with PMN suspension as detailed by Easmon et al (1980). Using different concentrations of serum resulted in varied chemiluminescence peaking times and responses (results not presented graphically); 40% serum concentration was the most effective amount for the assay. The effect of opsonized particle concentration was evaluated too using different amounts of zymosan. As illustrated in figure 31, light emission was greater if the opsonized particle concentration was increased up to 8 mg/ml but the peak response declined at 10 mg/ml.

Dose and time responses to a soluble stimulus

Phorbol myristate acetate (PMA), a cocarcinogen extracted from croton oil, is known to cause marked cellular oxidative metabolism of resting neutrophils (DeChatelet et al, 1976). Thus it forms a useful stimulus in the luminescent study of PMN. A stock solution of PMA (10^{-2} M) prepared in DMSO was further diluted to the desired strength suitable for stimulation. Similar procedural manipulations to that using opsonized zymosan were used. At 10^{-5} M a maximal peak response was achieved within a short period of time but above this concentration the DMSO was toxic to the cells preventing further investigation of the effects of higher PMA concentration on chemiluminescence response. Reducing the concentration of PMA resulted in a decline of the peak response and a later peak (Figure 32).

Discussion

The graphs in figures 31 and 32 depict the chemiluminescence patterns in response to both particulate and soluble stimuli. Further experiments showed that the peak response for PMA was generally much higher and occurred within a shorter period of time than the response to opsonised zymosan. The declining following the peak response also differed for both in that PMA showed a relatively rapid deceleration while opsonised zymosan had a gradual

decline. Contact of opsonised zymosan with the PMN cellular membrane results in an array of biological events: recognition via complement and IgG Fc receptors of the phagocyte, activation of cellular redox metabolism, phagocytosis, specific and azurophilic degranulation, and formation of phagolysosomes (Henson, 1971; Bainton, 1973). When PMA is used as a stimulus, there is activation of redox metabolism without true phagocytosis and this has led to a suggestion that PMA activates the cell in the same manner as does phagocytosis (DeChatelet et al, 1976). Later, protein kinase C was shown to be one of the key factor responsible in the activation of neutrophil function and that PMA is an exogenous activator of the same protein kinase (Wolfson et al, 1985). One drawback in the chemiluminescence study though is the variation in PMN response within normal individuals. Different batches of cells resulted in variations of the recorded values even though the pattern of response was preserved. Mean values for a normal standard curve are therefore impossible to tabulate.

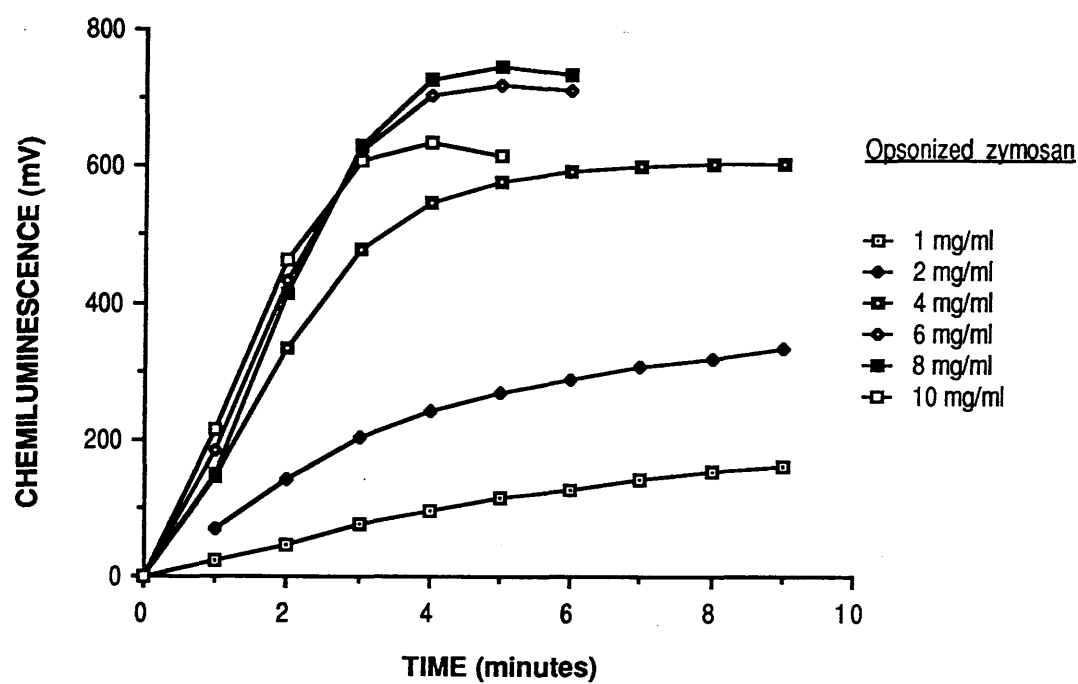


Figure 31. Dose and time responses to opsonized zymosan in the neutrophil chemiluminescent assay. Normal PMN (10^6 cells) were prewarmed in a 37° C water bath for 10 minutes before the addition of opsonized zymosan and luminol (10^{-4} M). The content was then placed in the luminometer to record chemiluminescence.

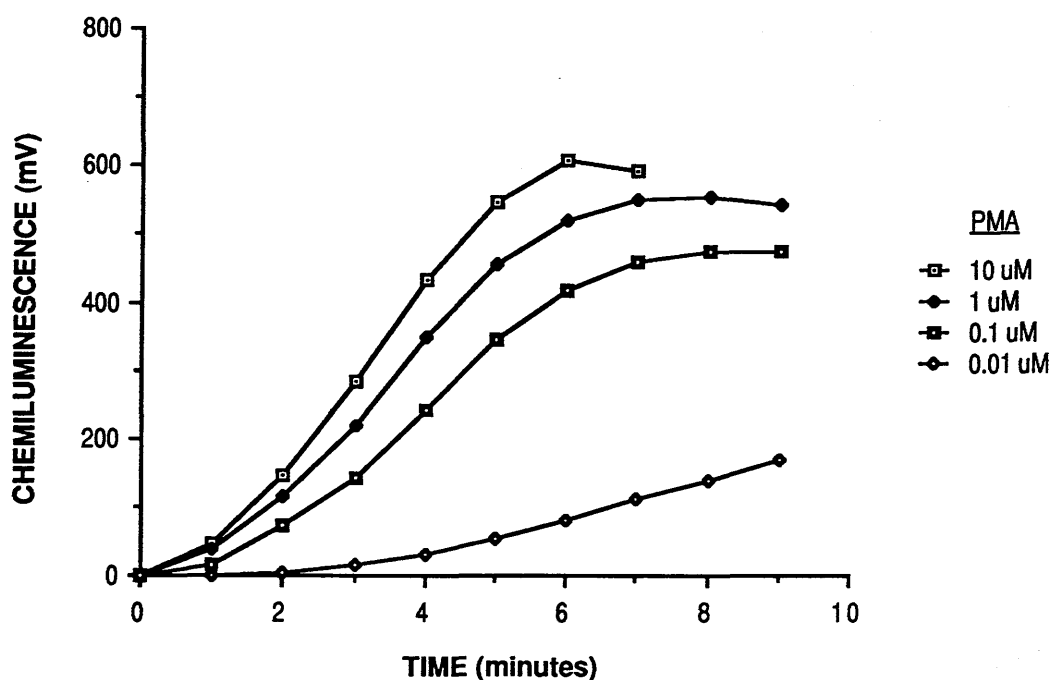


Figure 32. Dose and time responses to PMA in a chemiluminescent study. Normal PMN (10^6 cells/ml) were prewarmed in a 37°C waterbath for 10 minutes and then exposed to PMA in the presence of luminol (10^{-4} M) to record the chemiluminescence produced.

Section 11

CHEMILUMINESCENCE STUDY IN DIABETICS

Chemiluminescence provides a simple and sensitive means to assess the overall oxidative metabolic potentials of leukocytes. The quantitation of light from normal human neutrophils in response to a soluble and particulate stimulus is therefore useful before embarking on a comparative study on disease-state neutrophils such as that of diabetic cells.

Chemiluminescence using LPS with or without PAF

Normal or diabetic patient cells were mixed with 10 $\mu\text{g/ml}$ of LPS in a tube containing 10^{-4} M luminol and the response was recorded. In one experiment patient cells gave a very low peak reading of 45 mV after 72 minutes in comparison to normal cells peaking at 180 mV within 58 minutes (Figure 33). The initial response from the patient cells was also quite slow. Samples from different normal donors took 30 to 60 minutes to reach peak values with few exceptions (Table 7a) while it took between 60 to 100 minutes for patient cells to produce the maximum response (Table 7b). There were wide variations but the trend was that generally the normals produced greater chemiluminescence than the diabetics as confirmed by t-test statistical analysis (significant at $P < 0.05$). The blood glucose levels in these patients did

not seem to show any correlation with the low chemiluminescence response (Figure 34). In another set of experiments, both types of cells were preincubated with PAF at 10^{-8} M for 10 minutes before challenging with LPS and eventually used in a chemiluminescence assay. This was conducted since PAF has been reported to enhance neutrophils' oxidative responses to other stimuli (Ingraham et al, 1982; Dewald and Baggiolini, 1985; Gay et al, 1986). For normal cells there was a slight decrease in the response but an increase for the diabetic cells. Different batch of cells from both sets of donors showed a similar picture as graphed in figure 33 but with a wide variations in the responses (Tables 7a and 7b).

Chemiluminescence using LPS with PMA or opsonised zymosan

Normal PMN were preexposed to LPS for different times before introducing 8 mg/ml opsonised zymosan or 10^{-4} M PMA into the reaction tube. Figure 35 shows that in the absence of LPS the peak chemiluminescence response using opsonised zymosan was higher than those with LPS. Preincubation with LPS for 30 minutes gave little change, but incubation for 60 and 90 minutes resulted in a depression of chemiluminescence in response to opsonized zymosan. Using PMA as the stimulus, preexposure of the cells with LPS for

different times produced the opposite result to that obtained using a particulate stimulus. At all three periods of preincubation, LPS increased the cellular response to PMA (Figure 36); within 4 to 5 minutes the peaking point was reached while it took 7 minutes for the control to peak at a lower reading. Cells preexposed for 30 minutes responded better than those exposed for 60 and 90 minutes.

Assay on opsonised zymosan with or without agonist

The aforementioned procedural approach was used to compare the activity of the normal and diabetic cells. In figure 37, the first three bars starting from the left at each time sequence represent results of the assay using normal cells in the presence of opsonised zymosan with or without PAF or LPS. With the progression of time, addition of PAF caused an increase in the peak response from 605 mV to 800 mV without the agonist. The value was 563 mV with 30 minutes of preexposing LPS to the cells without PAF. The following three bars represent readings from patient cells assuming a similar pattern of response to the normal leukocytes. Even though the activity was still lower than the normal control the time required to reach the peak level was not different from that of the control (Table 8). Correlation test shows that no relationship exists

between blood glucose level and luminescence activity (Figure 38).

Assay on PMA with or without agonist

A very marked difference in the response was observed between diabetics and controls when PMA was tested (Figure 39). Preexposing PMN to LPS for 30 minutes and PAF for 10 minutes boosted the light emission from the normal and diabetic cells. Both types of cells responded more with the addition of LPS than PAF and again the patient cells showed no improvements in the readings in contrast to the normal donors (Table 9). However, a correlation test done on the patient samples indicate a positive relationship between the level of blood glucose and the chemiluminescence response towards PMA (Figure 40).

Discussion

Neutrophils generating light after phagocytosis of opsonised bacteria was first demonstrated by Allen and co-workers in 1972. Later chemiluminescence techniques were successfully employed for the assessment of PMN oxygenation activities in phagocytosis and microbial killing (Horan et al, 1982). One of the particulate stimuli that has been under discussion in inducing chemiluminescence in PMN is LPS. Conflicting results on whether bacterial LPS does induce light emission from

human PMN have been reported (Wilson et al, 1982; Henricks et al, 1983; Kapp et al, 1987; Pugliese et al, 1988). In this thesis by exposing the cells to LPS for over 90 minutes in a luminometer, chemiluminescence generation was detectable with the peak varying between 45 to 60 minutes depending on the donor cells. Direct activation of luminescence by LPS was observed though a long period of incubation was required. Beyond the 60 minutes mark, there was a slow decline in the activity. Such reactions were different using opsonized zymosan or PMA in both of which the response occurred within 10 minutes. As in the polarisation assay which took more than 60 minutes to maximise, there seems to be a slow activation by the LPS for both types of activities.

LPS has also been demonstrated to enhance neutrophil oxidative capacity upon subsequent contact with other stimuli (Guthrie et al, 1984). The effect known as priming may well be functioning when tested in chemiluminescence assay in which the response towards PMA was increased. Comparison of the priming effect was done with PAF which has been documented to prime neutrophils for enhanced release of superoxide anion (Worthen et al, 1988). The priming effect exerted by PAF was evident in that subsequent exposure with opsonised particles and PMA potentiated light emission, a result that differed from LPS actions. With opsonized zymosan the increase was greater for PAF than LPS, while using PMA the effect

induced by LPS was greater than PAF. Thus far the precise mechanism of LPS priming is unknown but the molecular basis of the enhanced activity has been investigated and related to changes in the NADPH oxidase and stimulus-response coupling (Forehand et al, 1989).

As mentioned earlier chemiluminescence assays have been employed to assess the normal and abnormal functional capacity of PMN in the production of oxygen metabolites. Patient cells from diabetic outpatient clinics were tested in the assay concurrently with normal controls. As expected and documented by other workers, light production using PMA and opsonised zymosan was impaired (Shah et al, 1983; Kjersem et al, 1988). The response of patients' cells to LPS was grossly impaired with a very slow and low activity. When the priming effect of LPS and PAF was studied, both exhibited similar results to that of normal cells: enhancement with PAF and LPS on PMA activation; depression with LPS on opsonised zymosan but not with PAF. Relationships between level of blood glucose and chemiluminescence response were negative except when PMA was used as the soluble stimulus (Figure 40). Small sample numbers could possibly result in a strong correlation between the two parameters.

The reason for the defective response in diabetics is still unclear but it seems that an intrinsic cellular defect of the PMN may be the key factor for the reduced

activity. In vitro experiments like chemiluminescence indicate the inability of diabetic cells to mount a normal oxidative burst which may contribute to the decreased intracellular killing by these leukocytes.

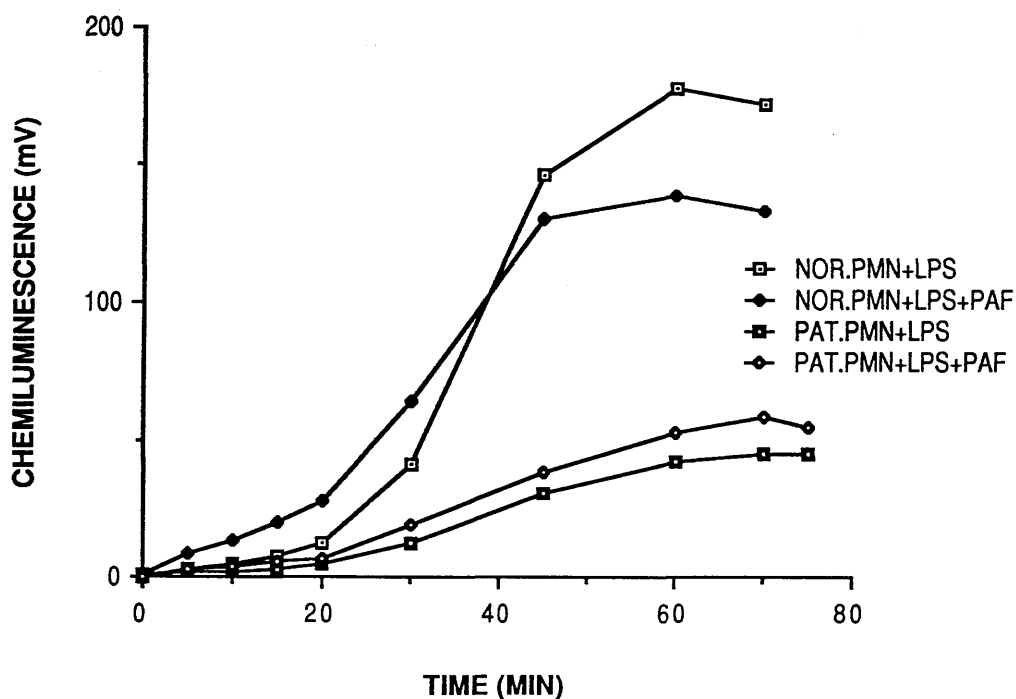


Figure 33. Chemiluminescent assay using LPS on normal and patients PMNs in the presence or absence of PAF. Cells at $10^6/\text{ml}$ were prewarmed in 37°C waterbath in the presence or absence of PAF (10^{-8} M) for 10 minutes after which they were exposed to LPS (10 ug/ml) to measure the chemiluminescence production. (NOR= normal cells ; PAT= patients cells)

Chemiluminescence with:

Donor	LPS		LPS + PAF	
	Peak response (mV)	Peak Time (min)	Peak response (mV)	Peak Time (min)
N1	131.2	62	118.1	55
N2	124.9	55	105.9	57
N3	98.2	57	83.6	50
N4	267.0	49	253.9	50
N5	170.0	32	158.5	40
N6	80.2	58	73.3	56
N7	143.8	67	157.1	70
N8	125.5	75	117.9	65
N9	63.9	96	61.4	90
N10	181.7	63	176.0	60
N11	115.4	62	104.9	59

Table 7a. Chemiluminescence assay on normal cells induced by LPS at 10 $\mu\text{g/ml}$ in the absence or presence of PAF at 10^{-8} M.

Chemiluminescence with:

Donor (BG)	Peak response (mV)	LPS Peak Time (min)	Peak response (mV)	LPS + PAF Peak Time (min)
P1 (14.4)	48.6	100	56.4	82
P2 (9.8)	47.2	80	62.1	62
P3 (10.3)	57.7	61	68.9	60
P4 (3.5)	40.8	90	60.5	75
P5 (15.3)	78.5	68	92.1	63
P6 (11.0)	46.2	70	59.9	65
P7 (12.0)	69.9	93	87.5	85
P8 (16.5)	34.4	60	45.6	52
P9 (5.1)	15.7	65	22.4	60
P10 (9.3)	99.7	72	125.6	67
P11 (9.8)	42.4	83	61.9	70

Table 7b. Chemiluminescence assay on patient cells induced by LPS at 10 $\mu\text{g/ml}$ in the absence or presence of PAF at 10^{-8} M. BG is the blood glucose level in mmol/lit. Comparison between normal (Table 7a) and patient cells using t-test shows a significant difference. $P < 0.05$

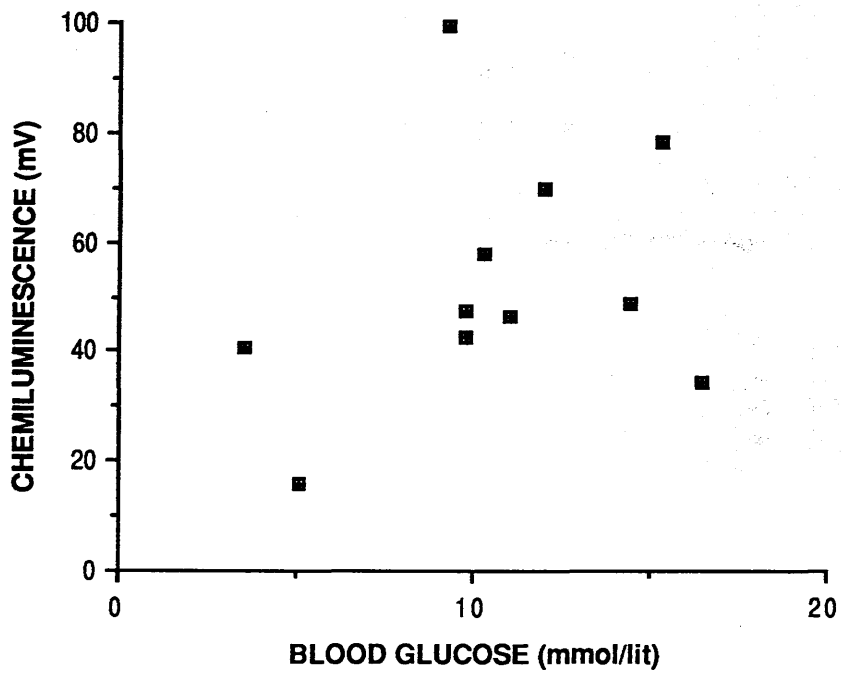


Figure 34. Comparison of chemiluminescence response of PMN in the presence of LPS with the level of blood glucose of insulin-dependent diabetic patients. (Correlation test, not significant, $P > 0.05$)

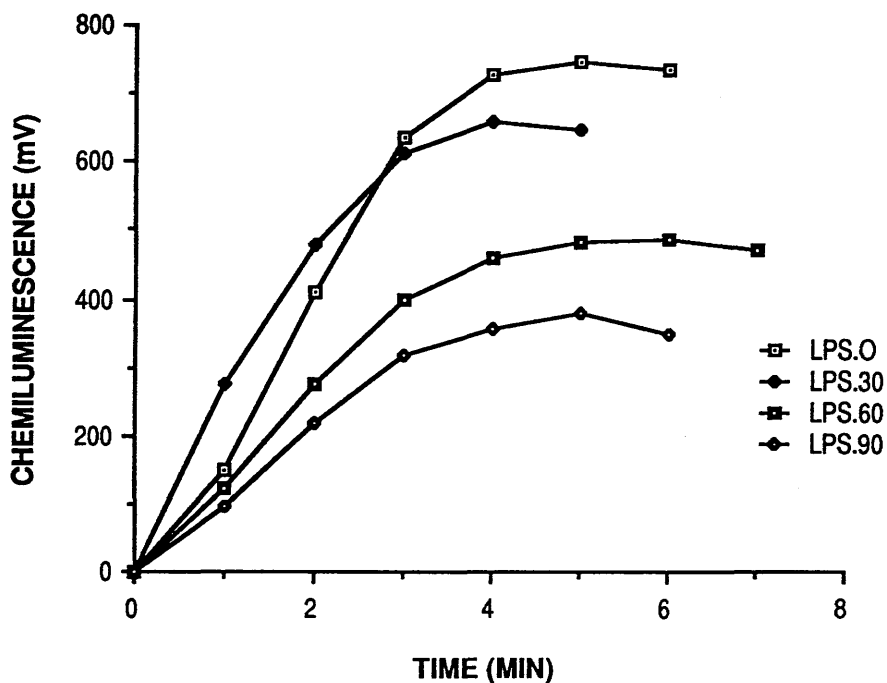


Figure 35. Chemiluminescent assay of the effect of opsonized zymosan on PMN preexposed to LPS. Normal PMN (10^6 cells/ml) were prewarmed in 37° C waterbath for 10 minutes before being exposed to LPS (10 μ g/ml) for 0, 30, 60 and 90 minutes. After each time interval opsonized zymosan at 8 mg/ml was added along with luminol to record chemiluminescence.

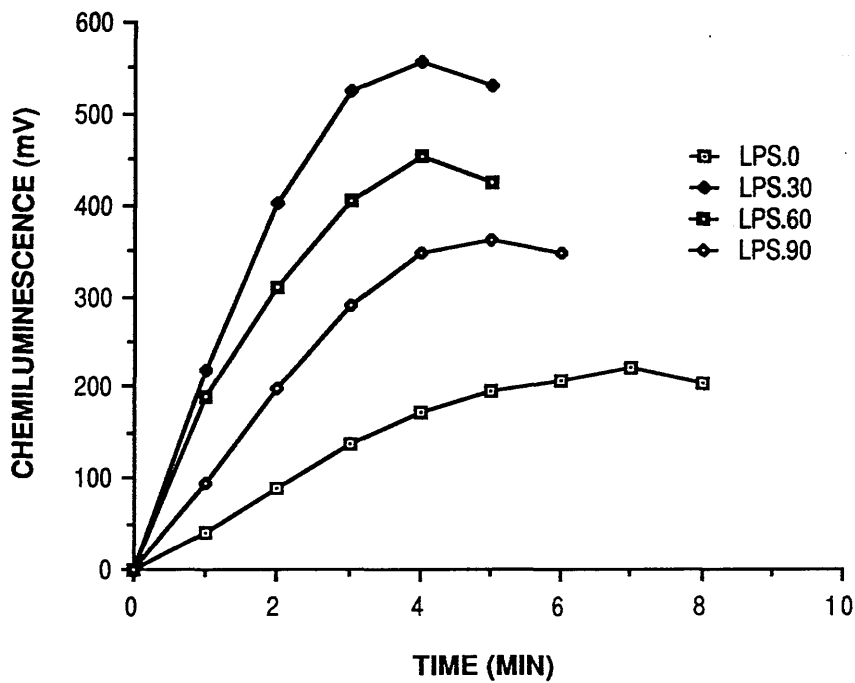


Figure 36. Chemiluminescent assay of PMA on PMN preincubated with LPS at various time periods. Normal PMN (10^6 cells/ml) were preincubated with 10 ug/ml LPS at various time periods after which PMA at 10^{-5} M was added at each interval .

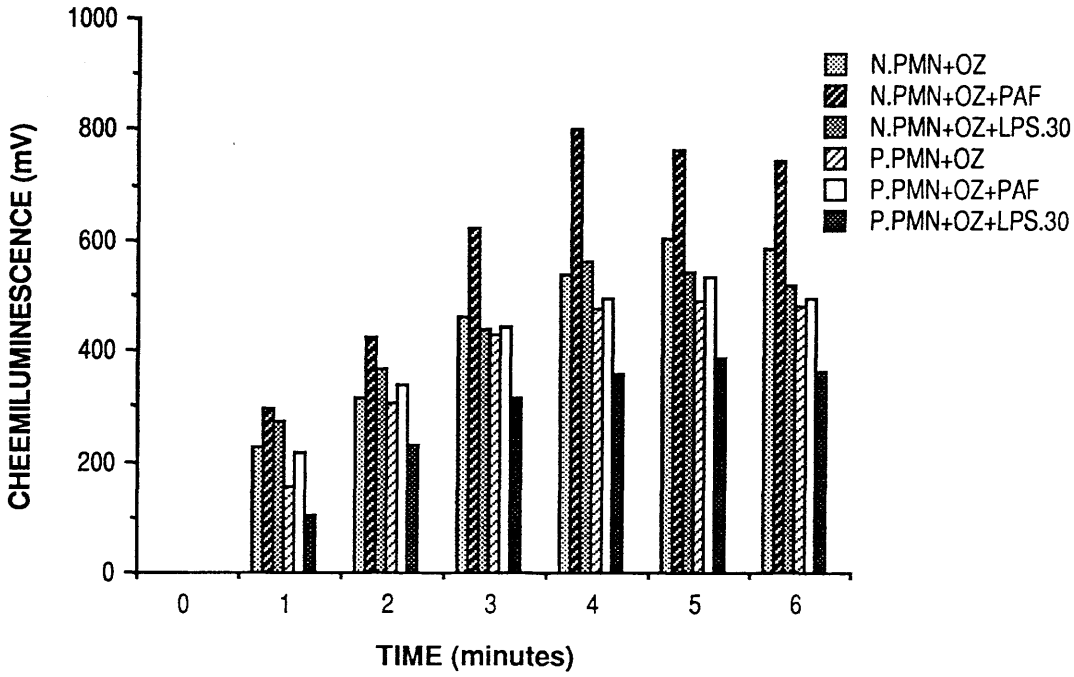


Figure 37. Chemiluminescent study of opsonized zymosan on normal and diabetics PMNs in the presence or absence of PAF or LPS. Cells at $10^6/\text{ml}$ were preincubated with PAF (10^{-8} M) for 10 minutes or LPS (10 ug/ml) for 30 minutes in 37°C waterbath. Opsonized zymosan (oz) at 8 mg/ml was then added to each content in recording the chemiluminescent produced. (N= normal cells ; P= diabetics cells)

Chemiluminescence with:

Donor	BG	OZ		OZ+PAF		OZ+LPS	
		Peak response	Peak time	Peak response	Peak time	Peak response	Peak time
P1	16.5	680.3	5	805	4	576.9	4
P2	5.1	545.4	6	575.7	4	487.9	4
P3	14.4	513	5	690	5	463.5	5
P4	12.2	745	5	833.6	5	657.2	4
P5	9.8	165.1	7	200	6	98.8	5
N1		783	7	912	5	662	6
N2		614	5	652	5	539	6
N3		548	6	709	6	484	5
N4		999	5	1082	5	793	5
N5		442	6	640	5	428	5

Table 8. Comparative study in chemiluminescence assay between normal PMN (N) and diabetic PMN (P) using opsonized zymosan (OZ) at 8 mg/ml in the absence or presence of PAF (10^{-8} M) or LPS (10 μ g/ml). BG is the blood glucose in mmol/lit, response in mV, and time in minutes.

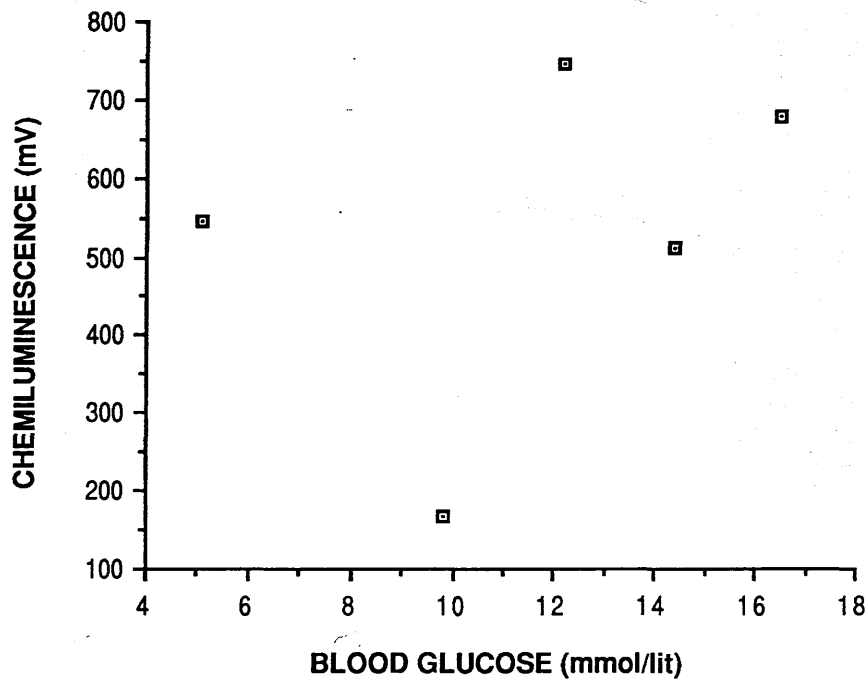


Figure 38. Comparison of the chemiluminescence response of PMN in the presence of opsonized zymosan with the level of blood glucose of insulin-dependent diabetic patients.

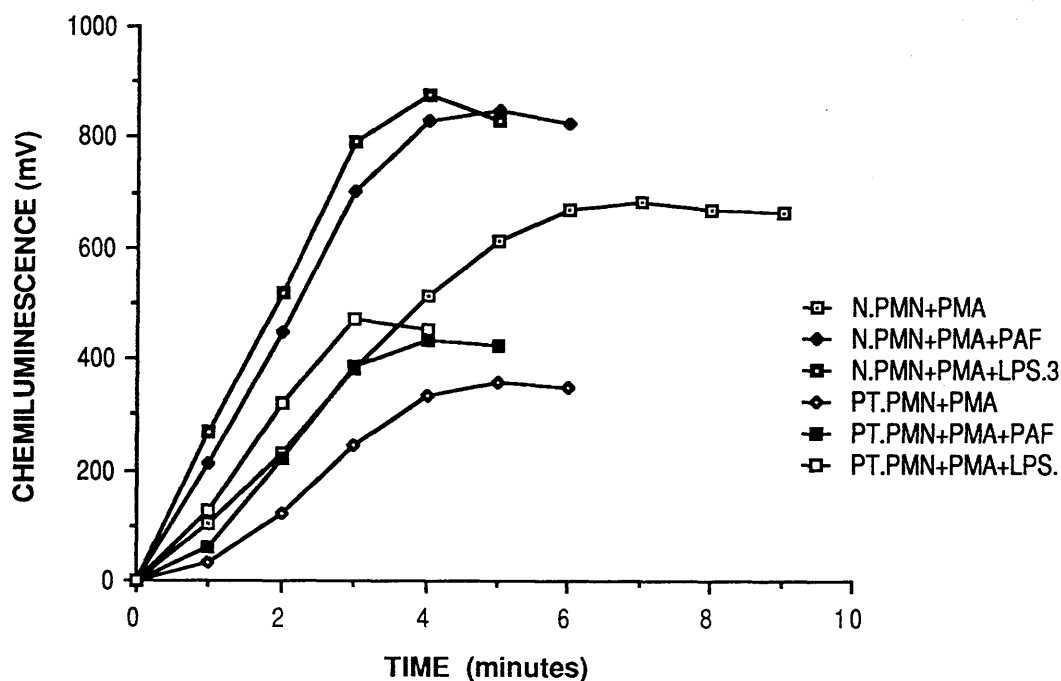


Figure 39. Chemiluminescent assay using PMA with or without PAF or LPS for normal and diabetics neutrophils. Neutrophils (10^6 cells/ml) were preincubated with PAF (10^{-8} M) for 10 minutes or with LPS (10 ug/ml) for 30 minutes in 37° C waterbath and later mixed with PMA at 10^{-5} to record the chemiluminescence. (N= normal cells ; PT= diabetics cells)

Chemiluminescence with:

Donor	BG	PMA		PMA+PAF		PMA+LPS	
		Peak response	Peak time	Peak response	Peak time	Peak response	Peak time
P1	12.4	357.2	6	534.1	3	612.0	4
P2	9.8	162.9	5	257.5	4	271.9	4
P3	14.0	510.4	5	581.4	4	711.0	4
P4	6.2	112.6	8	150.6	5	176.9	4
P5	10.2	162.7	7	214.7	5	220.3	4
N1		449.6	7	604.3	7	628.8	4
N2		587.8	8	655.0	7	705.5	5
N3		685.6	7	850.9	5	878.8	4
N4		336.0	5	574.6	4	605.0	4
N5		241.8	7	309.0	6	356.4	7

Table 9. Comparative study in chemiluminescence assay between normal PMN (N) and diabetic PMN (P) using PMA at 10^{-4} M in the absence or presence of PAF at 10^{-8} M or LPS at $10 \mu\text{g/ml}$. BG is the blood glucose in mmol/lit, response in mV, and time in minutes.

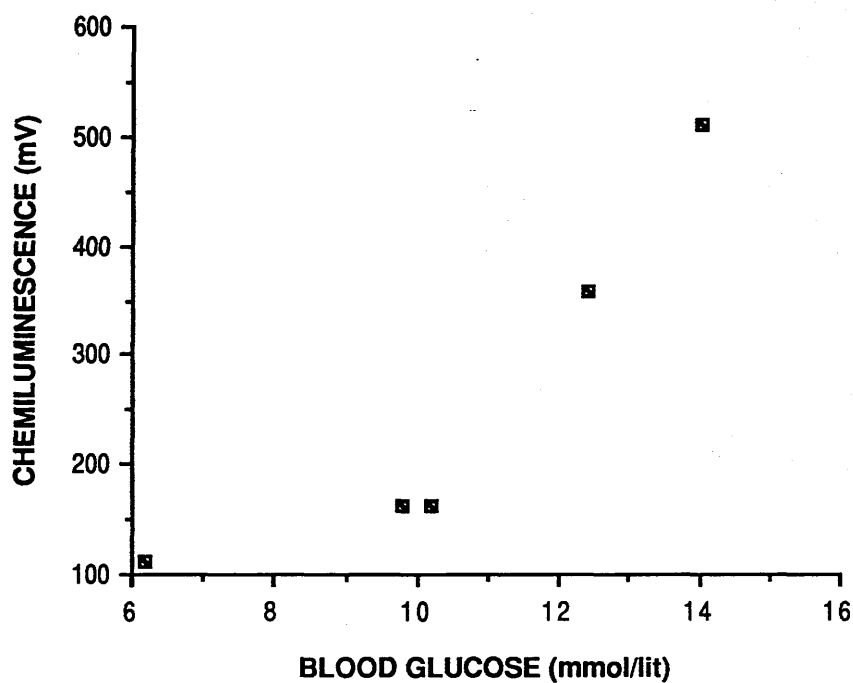


Figure 40. Comparison of the chemiluminescence response of PMN in the presence of PMA with the level of blood glucose of insulin-dependent diabetic patients. (Correlation test, significant, $P < 0.05$)

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

Accumulation of neutrophils in tissues is a prominent feature of inflammation observed in a variety of pathological conditions such as cancer, trauma and infections. The phenomenon of tissue infiltration is well characterised in bacterial infections whereby neutrophils are attracted in large numbers to eliminate the invaders. In other conditions neutrophils are recruited as scavengers of damaged tissues or unwanted extracellular deposits. Removal of foreign materials involves the production and releasing of bioactive compounds in particular required for the killing and digestion of microorganisms. However, these products also induce inflammation and tissue damage which normally appear after neutrophil accumulation.

In recent years several neutrophil chemoattractants have been isolated and well characterised each acting via unrelated receptors, suggesting that neutrophil recruitment can result from the concerted actions of multiple stimuli. Several functional responses to chemotactic stimuli including shape change, adherence, directed migration, enzyme secretion and the respiratory burst are demonstrated in vitro after stimulation. Non-chemotactic stimuli like LPS nevertheless do elicit such cellular functions. This is of importance as the neutrophil is one of the key elements in host resistance to bacterial infections. Current knowledge of the

effects of LPS derived from the outer cell envelope of gram negative bacteria on neutrophil function has been cited in the introductory part of the thesis. The accumulated data from previous studies support the view that neutrophils represent an important target for bacterial LPS. Besides altering blood levels of circulating neutrophils as reflected in leukocytosis or neutropenia, LPS are capable of influencing the locomotory, metabolic and bactericidal properties of PMN.

Polarisation and locomotion

Even though many researchers have investigated the effects of bacterial LPS on neutrophil functions as summarised by Wilson (1985), none actually investigated the possibility of neutrophils releasing a chemoattractant that may act on other neutrophils. What has been done in this project was to further resolve the question of what actually induced neutrophil shape change in the presence of LPS as discovered by Haslett and co-workers (1985). To assist such a study, the cell shape change assay or polarisation assay was implemented following the method of Haston and Shields (1985). The polarisation assay is quite useful for conveying information about the initial locomotory response but not about the locomotor events which follow polarisation. It permits the study of morphological

changes of cells when encountering any kind of a stimulant. The polarised form assumed by the cells was shown to be similar to that of cells in locomotion (Zigmond et al, 1981) but the adhesion of cells to surfaces may itself modify the spherical shape of PMN (Keller et al, 1979). Polarisation occurs independently of adhesion to the substratum (Keller and Cottier, 1981) thereby allowing in depth investigation of what induces shape change in suspension if the substance used is a non-chemotactic agent. Hence, with LPS the assay has been of great assistance in determining the cause of polarisation.

Before dealing with the unknown factor, the structure-function relationships of different LPS preparations should be discussed. Structural differences of different LPS preparations result in varied biological effects on PMN. To see if the structure of LPS does play a role in inducing shape change, different chemotypes of the bacterial LPS were examined. Results obtained clearly show that different forms of the LPS give different activity in the PMN response. Contradictory results reported from investigators, as reviewed in the introductory part of the thesis, using LPS to modulate PMN responses could be due to the different LPS preparations used. The most effective form was that of Re mutant strains which have been shown successfully to elicit better biological responses in

PMN such as the oxidative responses and inhibition of random migration than the other types (Kapp et al, 1987; Pugliese et al, 1988). The presence of the O core antigen was found to depress nonopsonic recognition of bacteria by phagocytes strongly (Wright and Jong, 1986). Modification of the LPS structure affects the interaction of bacteria with receptors on the phagocytes. When analysed, the Re chemotype has the outer sugar moiety removed consequently exposing the intact lipid molecule of native endotoxin which was and is still considered by the majority of experts in the field to be the only source of the reactions elicited by endotoxins both for its toxicity and beneficial effects (Nowotny, 1987).

In doing assays with LPS, polymyxin B is often added to in vitro samples to ensure that LPS activity is removed. Many investigators reported that polymyxin B could reduce the activity of endotoxin both in vivo and in vitro (From et al, 1979; Cooperstock and Riegler, 1981). Later many more investigators have suggested that polymyxin, either free or bound to a gel support, can be used to ensure a solution free of endotoxins (Duff and Atkins, 1982; Dinarello et al, 1984). Here in this work, inhibition by polymyxin B of LPS-induced polarisation of neutrophils was observed to be insignificant since the number of polarized PMN was minimally reduced. This ineffective inhibition could be due to the different

batch of polymyxin used than that of the above mentioned investigators. Failure of polymyxin to block the polarising activity could also be due to the presence of non-LPS agonists that might not interact with the antibiotic thus inducing polarization of neutrophils.

Cell-released chemotactic factors from neutrophils have been reported by earlier workers (Phelps, 1969; Borel, 1970; Zigmond and Hirsch, 1973). To document any release of a factor in a suspension of PMN, the supernatant was saved and tried on new batch of cells. Shorter onset times in responding towards the fluid fraction in comparison to that of exposing LPS directly implicates the presence of a factor other than the LPS itself. Though it was premature to accept such activity as being from a chemotactic factor, the similarity in time course of polarization to that of cells activated in the presence of classical chemoattractants such as FMLP was evident. Additional cell locomotion assays were needed to confirm that the factor was indeed behaving as a chemoattractant. As mentioned in previous chapters, a micropore filter assay gives additional information about the relationship between polarised morphology and cell motility since not all non-spherical leukocytes are motile and a direct test of locomotion is needed (Keller et al, 1985). Using the leading-front assay, PMN migrated deeper in the presence of the cell-release supernatant. Checkerboard studies were done to show

whether or not a directional response had been displayed by the cell population. The comparison of values between the experimental and the calculated figures as described by Zigmond and Hirsch (1973) point towards the existence of a chemotactic factor in the supernatant.

A collagen gel assay similar in principle to the micropore filter assay was simultaneously employed to study the response of leukocytes to a diffusing gradient of an attractant. The collagen gel assay has the advantage of not only allowing assessment of the locomotor capacity of cells in response to an attractant but also permitting direct observation of morphological changes of cells locomoting in a condition similar to what the cells experience in tissue matrices. Following the methodology developed by Islam and colleagues (1985), the collagen gel assay like the filter assay suggests that cells migrate towards the supernatant. The polarisation assay and the locomotion assays thus confirm each other. Visual assays fortify evidence for a cell-released factor. With a videotape recorder, cell movements in suspension after exposure to LPS were recorded and played back for analysis. Those polarised cells exhibiting crawling like movements in suspensions displayed good locomotion after settling down on the filming chamber, an observation similar to the findings of Keller et al (1983). The shapes of the polarised cells exposed to LPS were compared to those of cells

incubated with FMLP. Classical locomotor morphology was similar at later stages of the incubation with FMLP or LPS meaning that whatever induced the shape changes in the presence of LPS behaved in the same way as the chemoattractant FMLP. All techniques used to study and analyse cell locomotion suggest the presence of a factor released when cells were exposed to LPS for over 60 minutes.

When human neutrophils are exposed to appropriate stimuli, a complex sequence of events follows such as the generations of stable prostaglandins and release of leukotriene B₄ a known chemotactic factor (Goldstein et al, 1973; Samuelsson and Hammastrom, 1980). Since neutrophils are the primary participants in the inflammatory processes, the study of the effects of steroidal and non-steroidal antiinflammatory drugs (NSAID) on the cells responses are useful. These drugs slow down or eliminate the destructive effects of inflammation. Inhibitory effects of NSAID on neutrophil activation by a variety of ligands have been published by many investigators (Simchowitz et al, 1979; Kaplan et al, 1984; Minta and Williams, 1985). The involvement of arachidonic acid metabolites as activators of the shape change in leukocytes in this research is a distinct possibility. Using four different drugs to modulate the neutrophil polarisation activity, differences were noted: dexamethasone and indomethacin failed to inhibit

the polarisation activity while BW 755C and Revlon 5901A to a certain extent managed to block the release of a polarising factor.

Knowing that a stimulant must be present in the fluid fraction, it is of interest to pursue further studies to identify the substance. Straightforward determination of the physical properties of the factor suggests that the substance is composed of a low and high molecular weight molecules. Separating the two compounds needs a finer and more accurate instrumentation. Based on the gel filtration result, the major activity in the supernatant consists of a high molecular weight compound. A low molecular weight compound of about 1200 also contributed to the polarising activity of PMN. The fraction, hydrophobic on phase separation, could be derived from the arachidonic acid metabolism as evident by the interference of the released factor by the lipoxygenase inhibitors, BW 755C and Revlon 5901A. Characteristics of the fraction such as small size and heat stability infer that it may be a lipid molecule.

[Faint, illegible handwritten text follows]

All the experiments conducted establish that a factor is released by the PMN after prolonged exposure to LPS and that the factor can stimulate PMN locomotion

in the absence of serum. The requirement for the production of the polarising activities was an appropriate amount of LPS and the right cell concentration without any contamination from mononuclear cells. Activated mononuclear cells are likely to influence neutrophil functions at the sites of inflammation as well as in in vitro assays. Recently mononuclear cells cultured in the presence of LPS have been shown to ~~release~~ release factors capable of activating neutrophils (Schroeder et al, 1987; Walz et al, 1987; Yoshimura et al, 1987; Peveri et al, 1988). Production of this factor, later known as neutrophil-activating peptide-1/ interleukin-8, nevertheless needed more than three hours of incubation before any significant effects on the PMN biological status could be detected. Neutrophils releasing a factor after incubation with the isolated product obtained after preincubating macrophages with LPS need consideration since reports have shown that neutrophils could be "primed" by certain agonists like LPS and PAF (Guthrie et al, 1984; Vercelloti et al, 1988) thereafter enhancing subsequent cellular activation by another agonist. Priming is a phenomenon in which cells after pretreatment with an agonist are activated, maybe through modification of intracellular signals, to respond at increased intensity towards different agonists. Any trace amount of LPS present in the

supernatant could well be activating the PMN after the cells had made their first response to the released product. The activation of the cells then may arise from a combination of both factors secreted by the mononuclear cells and neutrophils. The polarising inducer in this project needs extensive characterisation using a more accurate methodology to identify the exact component of the substance. At present judging by the response of PMN, various endogenous chemicals could be released once PMN is being stimulated by LPS.

Receptor studies

Being a major target for the actions of bacterial lipopolysaccharides, it is assumed that the high sensitivity of the polymorphonuclear leukocytes to LPS derives from the receptors for LPS on the cell surface. Several investigators have shown that receptors for LPS might mediate the binding of bacteria to phagocytes (Tomita et al, 1981; Speert et al, 1984; Euteneur et al, 1986). The first reported finding on the receptor for bacterial LPS came from the work of Wright and Jong (1986) who were able to characterise the receptors responsible for the recognition of *Escherichia coli*. Further studies showed that LPS bind to CR3 (CD11/CD18) at distinct sites from that of C3bi (Wright et al, 1989). What makes the issue interesting is that the receptors belong to the family of receptors for

neutrophils adhesion to endothelial cells, the CD11/CD18 family of receptors. The expression of the functional CD18 complex molecules appears to be essential for the migration of the inflammatory cells since patients with a defective gene for the CD18 fail to mobilise leukocytes to sites of inflammation (Anderson and Springer, 1987) and infusion of anti-CD18 monoclonal antibodies into animals blocked the emigration of leukocytes into peripheral sites in response to noninfectious stimuli (Arfors et al, 1987; Rosen and Gordon, 1987). In vitro studies showed that CD18 molecules are necessary for the initial binding of leukocytes to the endothelium (Lo et al, 1989; Tonnesen et al, 1989).

With the available information, monoclonal antibodies against the CD11/CD18 family of glycoproteins were added to the polarisation assay system in the presence of LPS. The outcome of the tests indicates the existence of blocking effects of different degrees by the antibodies. More evidence of the blocking effects appeared when the supernatant tested in a short-term polarisation assay showed a reduction in the activity. Combination of the three monoclonal antibodies produced a greater blocking effect than the individual antibodies implying that all three members of the integrins may have participated in the recognition and consequently the binding of LPS molecules on the PMN cell surface.

Unlike the work of Wright and Jong (1986) which showed a strong contribution of the β chain to the specificity of the binding of LPS to the macrophages, here it appears that the binding site of the CD18 family is highly affected by the identity of the α chain. This difference could be attributed to the types of cells used and the antibodies tested may be of different activities. The participation of the α subunit in the polarization assay could also be the reason why the degree of inhibition differs for each member of the family since each has their own unique α -chain.

Human neutrophils express a heterodimeric receptor (CD11b/CD18) that has ligand binding specificity for the Arg-Gly-Asp (RGD) sequence within many adhesive proteins (Haynes, 1987; Wright et al, 1987; Russell and Wright, 1988). When peptides containing the RGD sequence were used to competitively inhibit binding of LPS to PMN, strong inhibition was observed for the pentapeptide and fibronectin analog despite the fact that these peptides are structurally different from LPS. The two ligands then appears to interact with the same binding site on the CD18 molecules. In contrast, separate binding sites were used to recognize the two classes of ligands were reported by Wright and colleague (1989).

Even though the outcome of receptor studies differs from those obtained by earlier workers, the common view is that a receptor for LPS binding on PMN cell surface

is required and its structural identity is becoming clearer. The receptors related to the CD18 family seems to have the ability to recognize more than one ligand. This ability is not unique to the integrins (Roth, 1988; Mowbray and Koshland, 1987) and therefore being a member of this superfamily, the CD18 family is predicted to possess such property.

Chemiluminescence studies

The other aspect of the biological response brought about by the interaction of LPS with PMN is the oxidative metabolism of the cell which generally leads to production of light. Reports published on the induction of neutrophil chemiluminescence by bacterial LPS were conflicting as cited in the previous chapters of the thesis. One possible reason for the varied results is the class of LPS used (smooth versus rough) as shown by Kapp et al (1987) who concluded that induction of PMN chemiluminescence by LPS is a property of R-form as S-form preparations are virtually devoid of this activity. Even though in this project only the rough form was used, the response was evident but after prolonged time of exposure. The period of exposure is therefore important since normal individuals show a range of 30 to 60 minutes of incubation time to attain the peak response while there are others who took longer to reach at that same level. Class type of LPS and the

time of exposure are two fundamental parameters that need considerable attention in doing the chemiluminescence study.

Besides exerting profound effects on the immune system, bacterial LPS have been shown to prime PMN such that the cells respond to subsequent stimulus with enhanced secretion of superoxide anion, acid hydrolases, elastase and PAF (Guthrie et al, 1984; Haslett et al, 1985; Fitschen et al, 1988; Worthen et al, 1988). In our chemiluminescence studies the priming produced different effects with different second stimuli (PMA or opsonized zymosan). One of the main variables that will affect the degree of priming is the time of exposure for it was noted that a longer period of exposure failed to potentiate the chemiluminescence activities on the soluble as well as the particulate stimuli. Previous workers obtained an optimal LPS priming after 60 minutes of incubation (Guthrie et al, 1984) but here 30 minutes of exposure was sufficient to produce a maximum priming effect. Subsequent stimulation by PMA was enhanced but not by the opsonized zymosan, an observation that requires further clarification. A clue to such a difference in the luminescence response may be gained from the work of Rosen et al (1989). In their work on macrophages it was shown that while treatment with LPS alone induced low level phosphorylation of the 68K protein (a protein found to be equivalent to the major

substrate for protein kinase C), it markedly increased the rate of subsequent PMA-dependent phosphorylation of this protein. This work was based on the observation that LPS potentiates protein kinase C-dependent signals in macrophages (Aderem et al, 1986). Though macrophages were employed in those studies, similar events would be expected to take place in other phagocytic cells including PMN. As a matter of fact a strong link between protein kinase C (PKC) and the activation of human neutrophils has been documented (Tauber, 1987). Opsonized zymosan stimulates superoxide anion release by a mechanism distinct from that of PMA. The response to a particulate stimulus is thought to be phospholipase A₂ mediated, calcium-dependent and independent of PKC while PMA bypasses these associated lipid metabolic activities directly stimulating the PKC (Maridonneau-Parini et al, 1986).

The role of PAF as a regulator of human neutrophil superoxide anion generation in response to soluble and particulate stimuli was also examined. In vitro, PAF has a stimulatory effect on human PMN (Shaw et al, 1981; Poitevin et al, 1984; Dewald and Baggiolini, 1986; Gay et al, 1986; Shalit et al, 1988). The present study suggests that PAF modulates neutrophil superoxide anion production in response to opsonized zymosan and PMA. Mechanisms through which PAF accomplishes enhancement remains unclear. PAF may represent another primary

oxidative stimulus acting through a priming effect similar to that of LPS via activating the PKC.

Studies in diabetic patients

Bacterial infections are more frequent and tend to follow a more severe course in patients with diabetes mellitus than in non-diabetics. Such observations have led investigators to postulate that infections in diabetics may be a consequence of neutrophil dysfunction since the microorganisms causing infections are often found to be of the same type isolated from patients with impaired PMN functions (Wheat, 1980; Wilson, 1986). In support of the hypothesis, neutrophil functions have been shown to be impaired (reviewed in the introductory part of the thesis) but the results from different laboratories are conflicting.

~~From~~ Deriving from the reports cited previously, neutrophils from diabetic patients would be speculated to be responding poorly towards bacterial LPS. Low polarisation activity noted from diabetic individuals of both IDDM and NIDDM types may indicate a less active involvement of the receptors in recognizing bacterial LPS. If, on the contrary, the recognition site for LPS is functioning normally it could mean that there is a failure to secrete the polarising factor. Oxidative bursts measured by chemiluminescence in the presence of LPS were apparently lower than the normal donors. Priming of the cells with PAF improves the response of

these cells on contact with LPS. PAF can induce an increase in expression of the CD11b/CD18 receptors on human neutrophils (Shalit et al, 1988). Taking account of this stimulatory effect of PAF on human PMN, it is probable that cell receptors towards LPS are being functionally expressed to produce a potentiating effect. The possibility thus is that either diabetic cells do not express sufficient numbers of cell surface receptors or biochemical activation in response to a stimulus is impaired. Presence of a priming substance switches on the functional abilities of these cells to a limited extent. Glycosylated haemoglobin and fructosamine were observed to be positively correlated with the degree of diabetes (Gonen et al, 1977; Graf et al, 1978; Paisey et al, 1980; Johnson et al, 1982). These biochemical parameters along with the blood glucose level were hence implicated to contribute to the impaired neutrophil functions in diabetic cells. Results from the project do not show any significant correlation between glycosylated haemoglobin and fructosamine and the assays conducted. The glucose level may on the other hand contribute to the defective function of PMN as observed in the in vitro study on the effect of exposing normal cells to glucose at various concentrations. All the experimental evidences may help to answer the question of a defective nonspecific defense mechanisms in diabetic patients.

Conclusion

Activation of the neutrophils in response to any stimulus may follow the general pathway of cellular signal transduction as proposed by Berridge and Irvine (1984). To explain what might have taken place in the response of PMN towards the presence of bacterial LPS, a diagrammatic presentation similar to the proposed response mechanism would be of a help (Figure 41). A possibility in this case is that the ligand, LPS, binds to the CD11/CD18 receptors at the external cell surface and leads to the breakdown of PIP_2 or phosphatidylinositol 4,5-bisphosphate which in turn triggers the activation of PKC. The activated PKC then generates signals, among them is release of a soluble factor. The released-factor then interacts specifically with a recognition site dissimilar to the LPS binding site. A molecular modification of the cell membrane presumably of the same kind to the initial coupling response occurs subsequently inducing a signal responsible for the alteration of cell behaviour.

Expression of the CD11/CD18 surface receptors on human neutrophils which encounter LPS would definitely be of importance in the inflammatory sites. Recruitment of other neutrophils to the site of infection would be one of the defense mechanism of PMN exposed to bacterial LPS. On the part of diabetic cells, the low or poor

biochemical aspects of cellular activation seem to be the key figure in these neutrophils displaying the abnormal phagocytic functions. Further investigations on the biochemical aspects of cellular activation may give a clue in correcting the defect in the patients' cells hence reducing the risk of bacterial infections in those individuals.

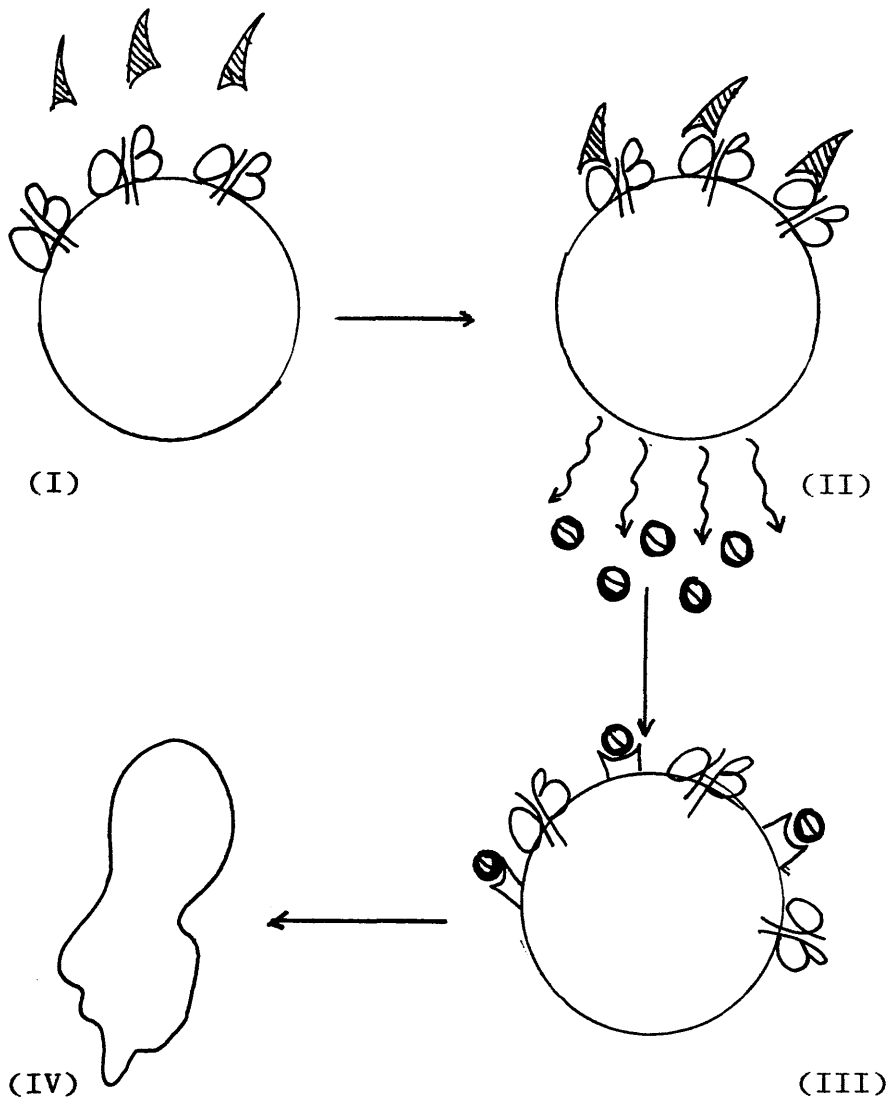


Figure 41. Diagrammatic presentation of PMN exposes to LPS.

- (I) PMN with surface receptors CD11/CD18 ($q\beta$) in suspension with LPS molecules (▲)
- (II) Binding of LPS molecules leading to the release of a factor (●)
- (III) Binding of the factor to specific sites (◻)
- (IV) PMN polarising

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2
This thesis is dedicated to my
mother and father
for all their love, support and encouragement.

